43. (Amended) A method of providing a [therapeutic] DNA, RNA, antisense RNA, ribozyme RNA, protein or peptide product to a patient in need of such product by administering to said patient an [a therapeutically] effective amount of the construct of [SV40 viruses or pseudoviruses according to] Claim 1.

44. (Amended) A method of providing a [therapeutic,] DNA, RNA, antisense RNA,[.] ribozyme RNA, protein or peptide product to a patient in need of such product by administering to said patient an [a therapeutically] effective amount of infected cells according to Claim 41.

45. (Amended) A composition [Pharmaceutical compositions] comprising as an active ingredient an [a therapeutically] effective amount of the construct of [SV40 viruses or pseudoviruses according to] Claim 1 in a pharmaceutically-acceptable carrier.

46. (Amended) A composition [Pharmaceutical compositions] comprising as an active ingredient an [a therapeutically] effective amount of the infected cells according to Claim 41, in a pharmaceutically-acceptable carrier.

REMARKS

Claims 1-46 are pending in the application. Claims 1, 6, 7-13, 15-19, 24-35, 40, 43-46 have been amended. Support for the amendments is described in the relevant sections below. No new matter is added by these amendments.

Drawings

The Examiner notes that the application has been filed with informal drawings. Applicants will file formal drawings upon indication of allowable subject matter.

Specification

The Examiner notes that the application does not contain an abstract of the disclosure as required by 37 C.F.R. § 1.72 (b). Applicants therefore submit herewith an abstract on a separate sheet as requested by the Examiner, and respectfully request that the following abstract be inserted into the application on the page following the claims:

--Methods for the *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous nucleic acids or exogenous proteins or peptides, and uses thereof are described.--

The Examiner notes that use of the trademark Baculogold™ is used in the application. Applicants have amended the specification as requested by the Examiner to indicate the proprietary nature of the product and to include generic terminology.

Claim Objections

Claim 1 is objected to because of informalities in that section "f)" should be broken into two sections. Applicants have corrected this claim as suggested by the Examiner and therefore respectfully request that the objection on this ground be withdrawn and reconsidered.

Claim 18 is also objected to because of informalities in that the claim should be amended to read "recombinant SV40 viruses". Applicants have amended this claim to provide proper antecedent basis, and therefore respectfully request that the objection on this ground be withdrawn and reconsidered.

Claim Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1-17 and 41-46 are rejected under 35 U.S.C. § 112, first paragraph as containing subject matter which was not described in the specification in a such a way as to enable one of ordinary skill in the art to which it pertains, or which it is most nearly connected, to make and/or use the invention.

Specifically, the Examiner states that these claims are drawn to a construct of SV40 viruses or pseudoviruses comprising exogenous nucleic acid, wherein the exogenous nucleic acid or an exogenous protein is therapeutic and therapeutic methods of using the construct. The Examiner states that "[w]hile applicants have shown *in vitro* constructs, they have not demonstrated *in vivo* therapeutic use for the construct," and that, "[i]n order to do so, undue experimentation is required." The Office Action then goes on to cite several references which recite difficulties encountered by others in the field of gene therapy.

The Examiner further stated that the nature of the invention is complex and that gene therapy is a new and developing art, and presented Marshall (*Science* 269:1050-1055, 1995), in particular page 1054, column 3, which states that: "[v]irus-based vectors have been the most efficient for inserting genes into cells in the lab, but they have run into problems in the clinic. Often, the fraction of cells receiving the new gene is low . . . . Boosting the rate of gene transfer

by increasing the concentration of vector or by dosing patients repeatedly may create another problem . . . ."

Applicants respectfully disagree with the Examiner, and believe that the claims as written are enabled. The standard for enablement under 35 U.S.C. § 112, first paragraph, is whether the claimed invention can be practiced without undue experimentation given the guidance presented in the specification and what was known to the skilled artisan at the time the subject application was filed. A specification which contains a teaching of how to make and use the full scope of the claimed invention must be taken as being in compliance with the enablement requirement of 35 U.S.C. § 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. *In re Marzocchi*, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971). See also M.P.E.P. § 2164.04. The Court of Customs and Patent Appeals has stated that:

> it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement.

Id. at 370 (emphasis original).

Applicants have shown *in vitro* constructed viruses and/or pseudoviruses comprising heterologous constituents such as DNA. The constructs of the invention have been shown to be very highly infective. The constructs did transmit DNA molecules which are biologically functional and did result in expression of the heterologous DNA in the infected cells. (see the Examples in the Specification, in particular, page 30, lines 1-10, and Figs 4a and 4b). These teachings enable one of ordinary skill in the art to extend the use of the *in vitro* constructed viruses and/or pseudoviruses to *in vitro* or *in vivo* use without undue experimentation.

Moreover, the Applicants have shown that the constructs of the invention can package very large plasmids (See Specification, page 19, lines 21-22 and page 20, lines 13-23). This feature is of importance in delivering large genes and regulatory and control elements, so no undue experimentation is needed for the packaging of large genes.

The Examiner appreciated in the Office Action that Applicants have provided guidance and working example for *in vitro* construction of virions and pseudovirions. However, it is not true that the guidance provided by the description for *in vivo* use is prophetic. This guidance is based on the teachings of the invention, which, combined with available information and

protocols, enables the *in vivo* use of the constructs of the invention without undue experimentation.

It is important to note that Applicants' invention claims constructs and methods of using "*in vitro* constructed" SV40 vectors which, after assembly, can be used in any method suitable for SV40 vectors constructed in cells using SV40 wild type helper virus. The *in vitro* method to prepare helper-free SV40 vectors as claimed in the present invention, allows the spontaneous self-assembly of pseudoviruses in the test tube without the involvement of the wild type SV40 virus. It has been demonstrated that SV40 pseudovirions can overcome the problems presented by the cited references, Marshall, Verma *et al.* (*Nature* 389:239-242, 1997), Orkin *et al.* (1995) and Anderson (*Nature* 392:25-30, 1998) as discussed in the following examples.

The hematopoietic compartment is a target of choice for the treatment of genetic deficiencies affecting blood cells, acquired diseases such as AIDS or various cancers. In cancers where hematopoietic cells are contaminated with cancerous cells, gene transfer can be used to purge the cancerous cells from the bone marrow prior to re-implantation in the treated patient. On the other hand, if the bone marrow is free of cancerous cells, then the goal is to protect it from the toxicity of anticancer drugs used in chemotherapy. Traditionally, the bone marrow is removed from the patient and re-implanted after the chemotherapy is performed. However, reconstitution of the hematopoietic compartment after re-implantation can take up to several weeks, leading to grave complications.

To solve this problem, the human multidrug resistance gene, *MDR*1, was cloned by Rund, D. *et al.* (*Human Gene Therapy* 9:649-657, 1998, Exhibit A). *MDR*1 was cloned into an SV40 pseudoviral vector containing the SV40 origin of replication (*ori*) and encapsidation signal (*ses*), and the plasmid was encapsidated in COS cells as SV40/*MDR*1 pseudovirions. Expression of the human *MDR*1 gene was demonstrated in murine MEL cells infected with SV40/*MDR*1 pseudovirions, using a monoclonal antibody (MRK16) specific for the human 170 kd P-glycoprotein. Functional P-glycoprotein was demonstrated by resistance to colchicine in NIH 3T3 cells infected with SV40/*MDR*1 pseudovirions. Activity of P-glycoprotein was assayed by rhodamine 123 dye exclusion and fluorescence-activated cell sorter analysis (FACS) in various cell types including hematopoietic cells. Highly efficient gene transfer and expression was demonstrated in all murine and human cell types tested, including primary human BM cells. Using multiplicities of infection (moi) of 1-2, over 95% of cells were found to become $MDR1^+$. The percent of $MDR1^+$ cells was proportional to the multiplicity of infection (moi). Rund *et al.*

concluded that the SV40 pseudoviral vector is efficient for gene transmission into human hematopoietic cells. Furthermore, they have shown that the *MDR*1 gene was stably expressed in transduced cells.

The technology and procedure of bone marrow transplantation in humans has been established for over 20 years, and autologous bone marrow transplantation is accepted to be a safe procedure. Thus, the studies of Rund *et al.* show that constructs based on the teachings of the present invention can be efficiently used in *ex vivo* gene therapy, using hematopoietic tissue.

In another recent example, Dalyot *et al.* (Dalyot-Herman, N. *et al.*, *J. Hematotherapy & Stem Cell Res.* 8:593-599, 1999, Exhibit B) showed efficient transduction of the human β-globin gene into human erythroid progenitors of patients with β-thalassemia, demonstrating the feasibility of using SV40 vectors for gene therapy of thalassemia. Using SV40 constructs, they investigated the expression of constructs carrying the human β-globin gene, where the constructs were delivered as SV40/β-globin pseudovirions. Expression studies were performed in primary human erythroid progenitors cultivated from peripheral blood of β-thalassemia patients, who are unable to produce normal β-globin RNA. This erythroid culture system recapitulates *in vitro* the process of growth, differentiation and maturation of authentic erythroid precursors, following induction by the addition of erythropoietin (Epo). Five days later the cells were infected with pseudovirions carrying the normal β-globin gene, and RNA was harvested on day eight. The results showed significant levels of transduced normal β-globin gene mRNA. A small DNA fragment derived from the 5' region of the HSII element of the human β-globin locus control region, LCR, enhanced expression of the linked β-globin gene 20-30 fold. The level of the exogenous normal β-globin mRNA expression was directly proportional to the multiplicity of infection. These studies demonstrate the feasibility of using the SV40/β-globin delivery system for *ex vivo* gene therapy of β-thalassemia.

In another example, BouHamdan *et al.* (BouHamdan, M. *et al.*, *Gene Ther.* 6(4):660-666, 1999, Exhibit C) demonstrated that delivery into human T-lymphocytes of an anti-integrase single-chain variable fragment (SFv) by SV40 provides durable protection against HIV-1. In those studies, an SV40 expression vector was developed to deliver SFv-IN (SV(Aw)). Expression of the SFv-IN was confirmed by Western blotting and immunofluorescence staining, which showed that more than 90% of SupT1 T-lymphocytic cells treated with SV(Aw) expressed the SFv-IN protein without selection. When challenged, HIV-1 replication, as measured by HIV-1 p24 antigen expression and syncytium formation, was potently inhibited in cells expressing

SV40-delivered SFv-IN. Levels of inhibition of HIV-1 infection achieved using this approach were comparable to those achieved using murine leukemia virus (MLV) as a transduction vector, the major difference being that transduction using SV40 did not require selection in culture, whereas transduction with MLV did require selection. Therefore, the SV40 vector as gene delivery system represents a therapeutic strategy for gene therapy to target HIV-1 proteins and interfere with HIV-1 replication. These studies demonstrate the feasibility of using SV40 vectors for *ex vivo* gene therapy of HIV.

There are many additional studies that demonstrate the feasibility of using SV40 for *in vivo* gene therapy. Strayer and Milano (Strayer, D.S. *et al.*, *Gene Ther.* 3:581-587, 1996, Exhibit D) have shown that SV40 vectors mediate stable gene transfer *in vivo* into a wide spectrum of tissues and organs. Tag^-, luc^+ SV40 DNA was constructed and used to prepare a replication-deficient SV40-derivative virus containing luc (SVluc). The ability of SVluc to transfer luc production *in vivo* was tested in two ways: (1) SVluc was inoculated into BALB/C mice intravenously, and (2) bone marrow cells treated with SVluc were infused into syngeneic hosts. Luc production was followed for 105 days by immunochemical analysis of peripheral blood and selected for 105 days by using anti-luciferase antibody, and by assay of luc enzyme activity in peripheral blood. Luc was found in 20-25% of peripheral blood nucleated cells from day 20 until 105 days or more. Luc-producing cells were also identified in liver, spleen, brain, kidney, skin and colon from day 20, also until 105 days or more. Analysis of whole blood showed fluctuating levels of functionally active luc enzyme beginning on day 21, and remaining substantially and significantly greater than control values to day 105. Thus, SV40 may transfer sustained expression of foreign genes to bone marrow and other organs for at least 3 months.

Additional studies demonstrated the efficacy of SV40 vectors in gene delivery and stable expression in the liver. Zern *et al.* (Zern, M.A. *et al.*, *Gene Ther.* 6:114-120, 1999, Exhibit E) demonstrated successful transduction and expression of an alpha 1-antitrypsin ribozyme in a human hepatoma-derived cell line, suggesting the use of SV40 vector for the treatment of alpha 1-antitrypsin (alpha 1AT) deficiency disease. This disease is one of the more common hereditary disorders that affects the liver and lung. The liver disease of alpha 1AT deficiency is generally thought to be caused by the accumulation of an abnormal alpha 1AT protein in hepatocytes, while the lung disease is thought to be due to a relative lack of the normal protein in the circulation. Therefore, one possible approach to prevent and treat alpha 1AT disease is to both inhibit the expression of the mutated alpha 1AT gene, and to provide a means of synthesizing the

normal protein. To do this, Zern *et al.* designed specific hammerhead ribozymes that were capable of cleaving the alpha 1AT mRNA at specific sites, and constructed a modified alpha 1AT cDNA not susceptible to ribozyme cleavage. Ribozymes were effective in inhibiting alpha 1AT expression in a human hepatoma cell line using an SV40 vector. In addition, the hepatoma cell line was stably transduced with a modified alpha 1AT cDNA that was capable of producing wild type alpha 1AT protein, but was not cleaved by the ribozyme that decreased endogenous alpha 1AT expression. These results suggest that ribozymes can be employed for the specific inhibition for an abnormal alpha 1AT gene product. The findings also suggest that SV40-derived vectors may represent a fundamental improvement in the gene therapeutic armarmentarium.

In another example, the use of SV40 vector to immunize against hepatitis B surface antigen by gene transduction was demonstrated by Kondo *et al.* (Kondo, R. *et al.*, *Gene Ther.* 5:575-582, 1998, Exhibit F). This study also showed that there was no immune response against the vector, and that repeated administration was possible. They reported successful immunization using an SV40-derived viral vector carrying Hepatitis B surface antigen (HBsAg) cDNA, SV(HBS). The vector was used to elicit anti-HBs. SV(HS) was injected intraperitoneally or subcutaneously into mice every 4 weeks. These mice were bled every 2 weeks and their sera assayed for antibody activity against HBsAg and SV40. Production of anti-HBs was measured by ELISA and confirmed by Western blot analysis, both of which demonstrated significant levels of anti-HBs after the second injection. We also tested production of anti-SV40 antibodies by the ability of sera to neutralize SV(HBS) infectivity. There was no evidence of neutralization of SV(HBS) infectivity even after eight inoculations. Thus, replication-incompetent SV40 is itself not a strong antigen. The data suggest that SV40-based transduction systems may be a useful vehicle for immunization and for other gene transfer applications when a need for multiple inoculations is anticipated.

Two additional studies, described below, demonstrated long-term amelioration of jaundice in Gunn rats (Parashar, B. *et al.*, *Hepatology* 28 (Suppl. 503A), #1364, 1998, Exhibit G; Parashar, B. *et al.*, *Hepatology* Suppl. 29 #551, 1999, Exhibit H).

A helper-free recombinant SV40, SV-hBUGT, and a recombinant SV40 expressing firefly luciferase (SV-luc) used as control, were infused into the portal vein of bilirubin-UGT-deficient jaundiced Gunn rats through an indwelling catheter. After 10-30 days, liver biopsy specimens from the SV-hBUGT-treated Gunn rats, but not from controls, were positive for the transgene (by DNA PCR), human bilirubin-UGT mRNA (by RT-PCR) and bilirubin-UCT

protein (by Western blot using a human bilirubin-UGT-specific monoclonal antibody). HPLC of bile showed excretion of bilirubin glucuronides (mainly monoglucuronide) in treated rats, demonstrating *in vivo* bilirubin-UGT activity. Mean serum bilirubin concentrations declined by 40% in the treated group in 2 weeks after the infusion and remained at that level throughout the study period (50 days). There was no evidence of antibody response against SV40 proteins or lymphocytic infiltration of the liver. Gene transfer experiments in cultured cells suggested random integration of the vector into the host genome. Parashar *et al.* have concluded that the recombinant SV40 is a non-replicative, non-immunogenic vector, that transfers genes efficiently into quiescent hepatocytes *in vivo*, and may represent a significant advance over current gene therapy vectors.

The second study was aimed at determining (a) whether rSV40 is immunogenic, (b) if rSV40-mediated gene transfer can be repeated and (c) whether the transgene integrates into the host genome. One, three or seven doses of SV-hBUGT (3.6'1010IU) were infused into the portal vein of Gunn rats through indwelling catheters, resulting in 40%, 65% and 70% reduction in serum bilirubin levels, respectively, which persisted throughout the period of observation (2 months). The rat sera were negative for antibodies against purified SV-hBUGT, as determined by ELISA and immunotransblot. There was no increase in serum alanine aminotransferase levels in the recipient rats and no lymphocytic infiltration was found in liver biopsy specimens, despite repeated injections of rSV40. To determine if the gene transfer can be repeated, an rSV40 expressing HBsAG was infused into the portal vein of naive rats or rats that had received 3 infusions of SV-hBUGT two weeks earlier. Immunoblot of liver homogenates showed equal level of HBsAg expression, demonstrating repeatability of gene transfer. Southern blot analysis of DNA extracted from livers of Gunn rats that had been treated with SV-hBUGT 3-5 months earlier showed that the transgene (h-BUGT) migrated with high molecular weight genomic DNA. The restriction analysis pattern indicated random integration of the transgene, and suggested that the recombinant viral genome is integrated as single copies, rather than as tandem inserts. Kadakol *et al.* have concluded that recombinant SV40 transfers transgenes efficiently into quiescent hepatocytes *in vivo*. The virus appears to integrate randomly into the host genome and is non-immunogenic, permitting long-term and repeatable gene transfer.

These studies all demonstrate that SV40 constructs can be used as a vector for *in vivo* gene therapy, including applications which require repeated administration, without undue

experimentation. Given the advantages of the *in vitro* constructed SV40 constructs of the present invention, they are particularly useful.

Furthermore, an important feature of the present invention is that it does not restrict the regulatory elements to be included in the constructs. This was a major limitation of viral vectors, which limited their application to gene therapy, as stated by Marshall. The present invention is unique in allowing inclusion of potent regulatory signals, as demonstrated by the inclusion of β-globin LCR elements (Table 3, page 35 of specification). Furthermore, the present invention has a large cloning space, which facilitates the inclusion of a combination of regulatory signals. This will ensure that the transduced therapeutic gene is appropriately expressed in the target tissue, as required for the treatment of the specific disease.

From all of the above, it is evident that the problems referred to by Marshall, Verma *et al.*, Orkin *et al.* and Anderson are not relevant to the constructs of the invention. In fact, notwithstanding such known reservations, the inventors have succeeded in constructing SV40 virions and pseudovirions that are infective and give expression in transfected cells of the heterologous DNA which the virions and pseudovirions contain.

Turning specifically to Verma *et al.*, these authors state on page 239, column 8, that "[t]he use of viruses is a powerful technique, because many of them have evolved a specific machinery to deliver DNA to cells. However, humans have an immune system to fight off the virus, and our attempts to deliver genes in viral vectors have been confronted by these host responses." While this may be a serious problem with many vectors, the SV40 vector was shown not to raise immune response and to allow repeated administration (Kondo *et al.*; Kadakol *et al.*).

The Examiner further refers to the box titled "What Makes an Ideal Vector" in Verma *et al.* The ideal vector, according to Verma *et al.*, should have high concentration, contain the appropriate integration and transcription signals, and should not elicit an immune response. The vectors of the present invention can be produced at high concentration, are capable of including appropriate site specific integration signals (such as the AAV inverted terminal repeats, ITR's), are capable of including appropriate regulation signals (as exemplified for the β-globin LCR) and, as already shown, SV40 constructs do not elicit an immune response (see, *e.g.*, Kondo *et al.* and Kadakol *et al.* (abstract), cited herein). Therefore, the invention overcomes the limitations presented by Verma *et al.* and provides an "ideal vector" for gene therapy.

Applicants have shown *in vitro* constructed viruses and/or pseudoviruses comprising heterologous constituents such as DNA. The constructs of the invention have been shown to be

very highly infective, and the constructs transmit DNA molecules which are biologically functional and result in the expression of the heterologous DNA in the infected cells. Armed with the Applicants' teachings in the specification, one of ordinary skill is able to extend the *in vitro* constructed viruses and/or pseudoviruses to *in vivo* use without undue experimentation. In addition, Applicants have shown that the constructs of the invention are capable of packaging very large plasmids. This feature is important in delivering large genes along with regulatory and control elements. The present invention is novel in that it allows inclusion of regulatory signals, as demonstrated by the inclusion of β-globin LCR elements (see Specification, Table 3). Furthermore, the present invention allows inclusion of a combination of regulatory signals, which insures that the transduced gene is appropriately expressed in the target tissue, as is required during treatment of a specified disease.

In order to ensure a speedy favorable outcome to prosecution however, Applicants have amended the claims to delete the term "therapeutic." Claims 43-46 now recite an "effective" amount of the construct. An effective amount of the construct can be an amount sufficient for the replication of a constituent, expression of a constituent or prevention of expression of an undesired protein or proteins in a cell. Applicants therefore respectfully request that the rejection on this basis be withdrawn and reconsidered.

Claims 14 and 15 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

Specifically, the Office Action states that the "specification does not sufficiently describe the possible inventions drawn from the claim", and that "there is no information given regarding the structures of a gene that may suggest potential antisense oligonucleotides which may be created or found." The Office Action goes on to state that "there are virtually an infinite number of such antisense oligoneucleotides of all potential target nucleic acids or their complements, and no disclosure as to which portions of the nucleic acids are structurally important . . . ." The Office Action then concludes that the specification fails to indicate that Applicants had possession of any of these compounds at the time of filing of the instant application.

Applicants are not required to reiterate that which is well known to one of ordinary skill in the art in order to be in possession of the claimed invention. As stated by the MPEP, "[a] patent need not teach, and preferably omits, what is well known in the art." (§ 2164.01).

Antisense is a very active field, and as of Applicants' filing date, numerous antisense patents had issued in the U.S., and many more technical references had been published. For instance, Martiat *et al.* (*Blood* 81:502-509, 1993, Exhibit I) describes selected antisense transcripts that are biologically active and inhibit expression of P210BCR-ABL, and Sczakiel *et al.* (*J. Virol.* 65:468-472, 1991, Exhibit J) teaches antisense RNA against HIV. A survey of the issued patents clearly indicates that successful antisense constructs need not be full-length, but only as long as necessary to allow hybridization to the native mRNA. An antisense nucleic acid can also be complementary to 5' or 3' untranslated regions, or overlapping the translation initiation codon (5' untranslated and translated regions). As few as 52 bases of 5' untranslated antisense RNA has been shown to inhibit enzymatic activity in some systems (Izant, J.G. *et al*, *Science* 229:345-352, 1985, Exhibit K). One wishing to make an antisense construct against a given nucleic acid therefore does not need to know which portions of that nucleic acid are structurally important, and Applicants therefore should not be required to point out such features. The precise sequence of an antisense construct will vary depending on the nucleic acid against which it is to hybridize, and the practitioner of ordinary skill will plan the antisense construct accordingly.

The enablement requirement of 35 U.S.C. § 112, first paragraph does not require Applicants to provide working examples in arts which are well known to those of ordinary skill in the relevant fields. Applicants respectfully submit that antisense methods are well-known, and that they should not be required to reiterate such methods. Applicants therefore respectfully request that the rejection on this basis be withdrawn and reconsidered.

Claim Rejections Under 35 U.S.C. § 112, Second Paragraph:

Claims 1, 3, 4, 7, 9, 10, 12, 25, 27-28, 32, 35, 37, 43 and 45 (and all dependent claims) are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention.

The Office Action states that Claim 1 is indefinite because it recites the phrase "capable of" which "renders the claims indefinite because the capacity of a compound to perform some function is merely a latent characteristic of said compound" and that such language "carries no patentable weight." Applicants are then referred to MPEP § 2173.05(b), (d) and (g).

Upon reading the cited sections of the Manual, Applicants respectfully disagree. These sections state that "[t]he fact that claim language, including terms of degree, may not be precise,

does not automatically render the claim indefinite under 35 U.S.C. 112, second paragraph" and that "[a]cceptability of the claim language depends on whether one of ordinary skill in the art would understand what is claimed, in light of the specification." (MPEP § 2173.05(b)). Applicants' Claim 1 recites a "construct capable of infecting a mammalian cell", and Applicants included the phrase "capable of" to indicate that it is desirable that the construct be able to infect a cell. A construct which is incapable of infecting mammalian cell is not useful in some of the embodiments of the invention, *e.g.*, Claim 41. That is, Applicants included the term "capable of" *precisely* for the purpose of particularly pointing out and distinctly claiming the subject matter which Applicants regard as the invention. Furthermore, Applicants can find no reference in the cited sections of the MPEP indicating that the phrase "capable of" carries no patentable weight, as stated by the Examiner. MPEP § 2173.05(g) even states that "there is nothing inherently wrong with defining some part of an invention in functional terms." This section goes on to provide an example where a limitation used to define a radical on a chemical compound as "incapable of forming a dye with said oxidizing developing agent", although functional, was acceptable because it set definite boundaries on the prosecution sought. Applicants' use of the phrase "capable of" in Claim 1 is anagolous to this accepted language, because it sets definite boundaries on the protections sought. Applicants therefore respectfully request that the rejection of Claim 1 on this basis be withdrawn and reconsidered.

The Examiner noted that the term "replication of a said exogenous protein" in Claim 1 was used to mean "that a protein can be replicated" while the accepted meaning is "that a nucleic acid can be replicated." Applicants actually meant "expression of said exogenous protein and/or replication of said nucleic acid". Applicants have amended Claim 1 to clarify the subject matter which Applicants regard as the invention. The amendment is supported in the specification on page 12, lines 1-2, and page 16, line 25 to page 17, line 18.

Claim 1 was also rejected under 35 U.S.C. § 112 second paragraph, the Examiner believing that "exogenous protein" in the phrase "replication of a said exogenous protein" has insufficient antecedent basis. Applicants have amended Claim 1 to remove the indefiniteness concerning replication of nucleic acid vs. expression of protein, and to therefore clarify the basis for "exogenous protein". Applicants therefore respectfully request that the rejection on this basis be withdrawn and reconsidered.

Claims 3 and 4 are also rejected for indefiniteness, the Examiner expressing a belief that a broad range or limitation together with a narrow range or limitation that falls within the broad

range or limitation within the same claim is considered indefinite. Claim 3 is rejected on this basis, the Examiner stating that Claim 3 recites the broad recitation "at least two" capsid proteins, and that Claim 1, from which Claim 3 depends, recites "at least one" capsid protein, which the Examiner believes is the narrower statement of the range/limitation. Claim 4 is similarly rejected, the Examiner believing that Claim 4 recites broad limitation of "a mixture of three" proteins, while Claim 1 (from which Claim 4 depends) recites the "narrower" range of "at least one" protein.

Applicants disagree. Claim 1 recites a construct comprising *at least one* semi-purified or pure SV40 capsid protein. This includes constructs containing one capsid protein, two capsid proteins, three capsid proteins, etc. Claim 3 on the other hand, recites the construct of Claim 1 comprising a mixture of *at least two* semi-purified or pure SV40 capsid proteins. This includes those constructs containing two capsid proteins, three capsid proteins, etc. The range recited in Claim 3 is therefore *narrower* than that recited in Claim 1, because the range in Claim 3 *does not include* those constructs containing only one capsid protein. Claim 3 therefore properly recites a further limitation on the range recited in Claim 1, rather than a broader range, as stated by the Examiner. The same is true for Claim 4, which recites "a mixture of three" proteins and therefore excludes two or one proteins, rendering Claim 4 narrower than Claim 1, which is as it should be.

Furthermore, the Board of Patent Appeals and Interferences stated in *Ex parte Wu*, (10 U.S.P.Q.2d 2031 (Bd. Pat. App. & Inter. 1989)) that "[i]n rejecting a claim under the second paragraph of 35 U.S.C. 112, it is incumbent upon the Examiner to establish that one of ordinary skill in the pertinent art, when reading the claims in light of the supporting specification, would not have been able to ascertain with a reasonable degree of precision and particularity the particular area set out and circumscribed by the claims." (*Id.* at 2033). The Board in that case found that use of the term "optionally" in a claim did not result in indefiniteness, stating that "the composition set forth in the claim can consist of the . . . . components recited or it can include . . . . a fourth component." (*Id.* at 2033). Applicants' Claim 3 is analagous to this situation in that one of ordinary skill in the art would understand that the construct of Claim 3 is meant to comprise a mixture of *two* or more capsid proteins, not merely one protein, as is allowed by the range recited in Claim 1.

Applicants therefore respectfully request that the rejection on this basis be withdrawn and reconsidered.

Claims 7, 10, 12, 25, 28 and 32 are rejected under 35 U.S.C. § 112, second paragraph as "being incomplete for omitting essential structural cooperative relationships of elements, such omission amounting to a gap between the necessary structural connections." Specifically, the Examiner believes that these claims are unclear as to whether the protein is normally not made by the intended host cell, or if the protein is not made in the cell when the DNA is contained within the cell. Applicants have amended these claims accordingly, and therefore respectfully request that the rejection of these claims on this basis be withdrawn and reconsidered.

Claims 7, 10, 12, 25, 28 and 32 are also rejected under 35 U.S.C. § 112, second paragraph, the Examiner believing that the terms "abnormally low amount," "defective form" and "physiologically abnormal or normal amount," are relative terms which render these claims indefinite. Applicants respectfully disagree. An Applicant is not required to define terms which are commonly understood, and absent definitions in the specification or claims, claim terms are to be interpreted according to their commonly understood meanings. The amount that is "normal" is either known to, or can be readily determined by, one of ordinary skill in the art. The term "abnormally low amount" is therefore to be understood as meaning precisely that -- an amount which is lower than that which is generally considered normal. Likewise, the term "defective form" is not indefinite, but is considered by one of ordinary skill practicing the invention as meaning forms that are nonfunctional. The term "physiologically abnormal or normal amount" is likewise not indefinite. Physiologically normal levels or amounts are known, or can be readily determined by one or ordinary skill in the art, and any substantial deviation would be considered "abnormal." Applicants have included with this Reply definitions of "abnormal," "defect" and "physiological." (*Stedman's Medical Dictionary*, 26th ed., Williams & Wilkins, Philadelphia, Pennsylvania, USA, pages 3, 447 and 1362, Exhibit L).

Because the terms cited by the Examiner are well within the knowledge of one of ordinary skill in the art practicing the invention, Applicants respectfully submit that these claims are clear on their face and are well within the understanding of one of ordinary skill. Applicants therefore respectfully request that the rejection on this basis be withdrawn and reconsidered.

Claims 9 and 27 are rejected under 35 U.S.C. 112, second paragraph, the Examiner believing that one of ordinary skill in the art would not know how to interpret the limitation "SV40 derived" in order to determine the means and bounds of the invention. Applicants disagree, and maintain that one of ordinary skill in the art would understand that the term "comprising SV40-derived *ori* DNA sequence" means *ori* DNA sequence from SV40, and that

the claims, as written, are clear on their face. Nevertheless, Applicants have amended these claims in the interest of clarity, to recite the term "comprising *ori* DNA sequence from SV40". The amendments are supported throughout the specification, particularly at page 17, lines 12-15, page 19, lines 17-20, and page 20, lines 19-22. Applicants therefore respectfully request that the rejection of these claims on this basis be withdrawn and reconsidered.

Claim 35 is rejected under 35 U.S.C. § 112, second paragraph, due to insufficient antecedent basis for the term "recombinant." This claim has been amended to recite the "*in vitro* constructed SV40 pseudoviruses" of the first line of this claim as originally filed. This claim now has proper antecedent basis, and Applicants therefore respectfully request that this rejection be withdrawn an reconsidered.

Claims 43 and 45 are rejected under 35 U.S.C. § 112, second paragraph, there being insufficient antecedent basis for the term "SV40 viruses or pseudoviruses". These claims have been amended to recite the construct of Claim 1 to provide such antecedent basis, and Applicants therefore respectfully request that the rejections on this basis be withdrawn and reconsidered.

Claim Rejections Under 35 U.S.C. § 102(b)

Rejection of claims as anticipated by Forstova et al.

Claims 18, 19, 21, 22, 24, 29 and 30 are rejected under 35 U.S.C. § 102(b) as being anticipated by Forstova *et al.* (*Hum. Gene Therapy* 6:297-306, 1995). The Examiner states that Forstova *et al.* teach a method of construction of SV40 viruses and pseudoviruses comprising a semi-purified or pure SV40 capsid protein, where the capsid was assembled and then the exogenous DNA was added to give pseudoviruses, etc. The Examiner states that Forstova *et al.* teaches each and every aspect of the instant invention, thereby anticipating Applicants' invention.

Applicants respectfully disagree. Applicants have read the cited reference, which concerns murine polyoma viruses, not SV40 viruses. Applicants were unable upon careful reading, to find any mention SV40 viruses within this reference. All of Applicants' claims recite SV40 viruses, pseudoviruses, or proteins therefrom. Applicants' invention is not anticipated by Forstova *et al.* because (i) polyoma and SV40 are not the same virus, and (ii) the experiments described by Forstova *et al.* do not lead to true packaging of DNA inton pseudovirions.

Polyoma and SV40 are not the same virus.

The Examiner stated that Forstova *et al.* taught a method of construction of SV40 viruses and pseudoviruses comprising a semi-purified or pure SV40 capsid protein, where the capsid was assembled and then the exogenous DNA was added to give pseudoviruses. Forstova *et al.* are not concerned with SV40 virus, but with polyoma virus.

Forstova *et al.* describe the use of polyoma virus "pseudocapsids", and not of SV40 *in vitro* constructed viruses or pseudoviruses, as carriers of heterologous DNA into mammalian cells. Although SV40 and polyoma are both members of the papova virus family, applicants respectfully wish to stress that polyoma and SV40 are two distinct, different viruses.

Importantly, polyoma and SV40 do not attack the same hosts. Polyoma is a murine virus whereas SV40 is a primate virus, found in nature in monkeys and capable of infecting human cells. As explained by John Tooze (Tooze J., *DNA Tumor viruses*, 2nd Ed., Part 2, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1981, p. 12, Exhibit M), "[s]ome cells (e.g., mouse cells including those of stable lines such as 3T3) are nonpermissive for SV40 but permissive for polyoma virus; other cells (e.g., hamster cells) do not allow either virus to replicate well. However, cells permissive for both SV40 and polyoma virus have never been described."

Both viruses are also quite distinct at the molecular level. The early proteins of polyoma comprise three T-antigens while SV40 has two. Middle T-antigen of polyoma is its transforming protein, and polyoma large T is required for viral DNA replication. In SV40, there is no middle T. Large T-antigen is required for viral DNA replication and also functions in immortalizing the host cell. The late proteins are also different. Polyoma capsid proteins VP2 and VP3 are smaller than SV40 VP2 and VP3, respectively. Polyoma VP2 and VP3 lack a DNA binding domain that is present in SV40 VP2 and VP3.

The structure of the regulatory region is also different. The polyoma enhancer is required for polyoma DNA replication, while the SV40 enhancer is not required for SV40 DNA replication. SV40 has a packaging signal, *ses*, while polyoma has no signal for packaging.

It therefore can be concluded that polyoma virus cannot be considered as a model for the SV40 vector. Therefore, the present invention is not anticipated by the experiments described by Forstova *et al.*

The experiments described by Forstova et al. do not lead to true packaging of DNA into pseudovirions.

The Examiner stated that Forstova *et al.* taught the assembly of the capsid and then adding the exogenous DNA to give pseudoviruses, and that these pseudoviruses were suggested to be used as a gene therapy delivery vehicle. Applicants respectfully disagree because the teachings of Forstova *et al.* do not lead to true pseudoviruses.

In their first experiment, Forstova *et al.* demonstrated, by sucrose gradient sedimentation, association between the polyoma "pseudocapsids" and a short DNA fragment of 1.7 kb. They subsequently showed that only 5-10% of that DNA that is found in association with the "pseudocapsids" is protected from DNase I digestion. These results demonstrate that the association between DNA and the polyoma "pseudocapsids" is not true packaging but some different kind of association which confers minimal protection against DNase I.

In their second experiment, they attempted to package circular supercoiled plasmid DNA of 6.2 kb in size. Their results (Fig. 3B) showed that only a linear fragment of about 2 kb became protected from DNase I digestion, proving that the polyoma "pseudocapsids" do not truly package DNA. Furthermore, there is no characterization of the 2 kb fragment which was protected from DNase I digestion. It may well be a random 2 kb digestion product of the original plasmid.

Therefore, it is respectfully argued that the work of Forstova *et al.* describes uncharacterized complexes of polyoma pseudocapsids with limited size linear DNA, and does not lead to the production of infective pseudovirions. The work of Forstova *et al.* does not teach the packaging of infective pseudovirions. The present invention, which enables packaging of intact supercoiled plasmid DNA into infective SV40 pseudovirions, is not anticipated by Forstova *et al.*

As stated by the Federal Circuit, "[t]o anticipate a claim, a reference must disclose every element of the challenged claim and enable one skilled in the art to make the anticipating subject matter." (*PPG Industries, Inc. v. Gurdian Industries Corp.*, 75 F.3d 1558, 37 U.S.P.Q.2d 1618 (Fed. Cir. 1996), at 1624). Because Forstova *et al.* fails to make any mention of SV40 viruses, pseudoviruses or proteins therefrom, which are integral to Applicants' claims, this reference cannot anticipate Applicants' invention. Applicants therefore respectfully request that the rejection on this basis be withdrawn and reconsidered.

Rejection of claims as anticipated by Christensen et al.

Claims 18-22 are rejected under 35 U.S.C. § 102(b) as being anticipated by Christensen *et al.* (*Virology* 75:433-441, 1976). The Examiner states that Christensen *et al.* teaches a method of construction of SV40 viruses and pseudoviruses comprising a semi-purified or pure SV40 capsid protein and at least one other SV40 protein, etc., and that this reference teaches "each and every aspect of the instant invention, thereby anticipating Applicants' invention."

Applicants disagree. Christensen *et al.* describes disruption of SV40 virions and the formation of "infectious DNA-protein aggregates," and not the packaging of SV40 pseudovirions, as described in the present invention. Christensen *et al.* describe aggregates comprising SV40 proteins and SV40 DNA. These aggregates, therefore, comprise SV40 proteins and nucleoprotein complexes, and not the packaging of naked, heterologous, supercoiled DNA. Christensen *et al.*, therefore, fails to anticipate Applicants' invention.

In addition, the Christensen *et al.* reference teaches the disruption at pH 10.6 of complete SV40 virions, which were then reassociated by HCl neutralization into a heterogeneous group of DNA-protein aggregates. The article by Christensen *et al.* describes disruption of SV40 virions and the formation of "infectious DNA-protein aggregates," and not the packaging of SV40 pseudovirions, as in the present invention. Christensen *et al.* describe aggregates comprising SV40 proteins and SV40 DNA. These aggregates comprise SV40 proteins and nucleoprotein complexes, and not the packaging of naked, heterologous, supercoiled DNA, all important elements of the present invention. In addition, the formation of the aggregates does not represent true DNA packaging, therefore pseudoviruses are not taught. For all these reasons, this publication does not anticipate the present invention.

The experiments described in Christensen *et al.* clearly demonstrate, as stated by those authors, that "these aggregates are not physically identical to complete SV40 virions" (page 433. right-hand column). The disruption of SV40 virions by alkali treatment (pH 10.6) is only partial; the DNA remains associated with the proteins, and both sedimented together as 110S (under the same conditions, free DNA remains at the top of the gradient). These partially disrupted virions were allowed to re-associate by lowering the pH to 8.0. The aggregates did not sediment in sucrose gradients as SV40, with a sharp peak of 240S, but produced a broad peak in the range of 150-205S (see Fig. 2). These findings show that the aggregates are physically different from complete SV40 virions.

While DNA which is complexed with capsid aggregates, as described in Christensen *et al.*, may be more effective than naked DNA, such aggregates will be readily degraded *in vivo* due to dissociation, and degradation of both the DNA and capsid proteins. Under the same conditions, pseudovirions that are properly assembled to reassemble the wild-type virus, as in the present application, will survive.

Measurements of the relative ratio of proteins (labeled by ^{14}C) to nucleic acids (labeled by ^{3}H) showed that the aggregates were chemically different from SV40 virions. The ratio of ^{3}H to ^{14}C in SV40 before disruption was 2.9, while the ratio in the reconstituted aggregates was only 1.2 (see Table 1), proving that the aggregates had a very different composition. Furthermore, the ratio of ^{3}H to ^{14}C of the disrupted virions was also 1.2, indicating that the difference between "disrupted virions" and "reconstituted aggregates" was not in their chemical content. The "reconstitution" achieved was not the packaging of DNA in protein shells, but a conformational change of pre-existing aggregates.

In addition, the title and the abstract of this reference state that these aggregates are infectious, but careful reading (see especially page 437, first column, lines 2-7), shows that these reaggregated protein-DNA complexes exhibited extraordinarily low rates of infectivity, namely 0.36 % of the initial PFU (plaque forming units), or a 280-fold loss of infectivity of the particles.

It is thus evident that the study of Christensen *et al.* is not on packaging of DNA, neither do the authors claim that it is. Therefore, the article of Christensen *et al.* is not prior art relevant to the present invention.

Furthermore, the infectious aggregates formed by Christensen *et al.* are not *in vitro* packaged virions. To explain the infectivity of the reconstituted aggregates, Christensen *et al.* suggested that the aggregates "can act to transport the DNA and enable it to penetrate the susceptible cell more readily than if the DNA were acting alone" (page 439, bottom of right-hand column) or, that "[i]t is likely that the capsid material is acting as a carrier facilitating the entrance of the DNA into the cell, thereby increasing the efficiency of infectivity" (page 440, bottom of left-hand column). Recent studies provide strong support for this explanation:

a. The SV40 capsid proteins recognize MHC class I receptors at the cell surface and facilitate SV40 entry (Norkin, L.C., *Immunol. Rev.* 168:18-22, 1999, Exhibit N; Parton, R.G. *et al.*, *Immunol. Rev.* 168:23-31, 1999, Exhibit O).

b. The SV40 capsid proteins bind DNA at a high affinity (VP1: Soussi, T., *J. Virol.* 59:740-742, 1986, Exhibit P; VP2, VP3: Clever, J. *et al.*, *J. Biol. Chem.* 268:29877-20883, 1993, Exhibit Q).

c. The SV40 capsid proteins have each a nuclear localization signal (VP2, VP3: Gharakhanian, E..*et al.*, *Virology* 178:62-71, 1990, Exhibit R; VP1: Ishii, N. *et al.*, *J. Virol.* 70:1317-1322, 1996, Exhibit S).

d. Association with capsid proteins promote nuclear targeting of DNA (Nakanishi, A. *et al.*, *Proc. Natl. Acad. Sci. USA* 93:96-100, 1996, Exhibit T).

From all of the above it is evident that the aggregates formed by Christensen *et al.*, in which SV40 DNA is complexed in an uncharacterized fashion with SV40 capsid proteins, may deliver the complexed SV40 DNA into cells and may target the DNA into the nucleus, rendering it infectious. However, Christensen *et al.* do not teach how to deliver heterologous genes and foreign regulatory sequences. Furthermore, Christensen *et al.* do not teach how to construct SV40 pseudovirions, carrying heterologous DNA. Therefore, it is evident that the infectious aggregates formed by Christensen *et al.* are not *in vitro* packaged virions. The article of Christensen *et al.* does not constitute prior art relevant to the present invention.

Finally, Applicants' Claim 18 recites a method for the construction of recombinant SV40 viruses or pseudoviruses comprising exogenous nucleic acid such as heterologous genes and foreign regulatory sequences. Applicants have been unable to locate that portion of the Christensen *et al.* reference that discloses that exogenous nucleic acid was included in their reconstituted virions. "To anticipate a claim, a reference must disclose every element of the challenged claim and enable one skilled in the art to make the anticipated subject matter." *PPG Industries, Inc. v. Guardian Industries Corp.*, 75 F.3d 1558, 37 U.S.P.Q.2d 1618, 1624 (Fed. Cir. 1996). The Christensen *et al.* reference does not teach one of ordinary skill to make recombinant SV40 viruses or pseudoviruses containing exogenous nucleic acid, i.e., in vitro packaged virions. Because the reference does not disclose each and every element of Claim 18, Applicants respectfully request that the rejection on this basis be withdrawn and reconsidered.

Claim Rejections Under 35 U.S.C. § 103(a)

Rejection in view of Forstova et al. or Christensen et al. in view of Carswell et al., Oppenheim et al. and U.S. Pat. No. 5,863,541

Claims 23, 25-28 and 31-38 are rejected under 35 U.S.C. § 103(a) as being unpatentable over the above-cited references.

As described above, Forstova *et al.* makes no mention SV40 viruses, and Christensen *et al.* were unable to produce infectious DNA-protein aggregates that contained intact DNA.

U.S. Pat. No. 5,863,541 teaches the production of AAV capsids which may be used to transfer native or heterologous molecules into appropriate host cells. This reference does not teach construction SV40 viruses or pseudoviruses comprising exogenous nucleic acid or exogenous proteins or peptides, nor does it make any suggestion to do so. It teaches methods of producing capsids for a completely different type of virus. SV40 is a member of the papova family and belongs to Class I, which is composed of the double-stranded circular DNA viruses. AAV is a member of the replication-defective *Dependovirus* genus of the parvovirus family, which belongs to Class II, which is composed of single-stranded linear DNA viruses. SV40 is an autonomous virus, capable of infecting the host and propagating on its own. In contrast, the members of the *Dependovirus* genus of the parvovirus family are entirely dependent on adenovirus or herpesvirus superinfection for the provision of further helper functions essential for their replication. The help required appears to be for a modification of the cellular environment (probably affecting transcription of the defective parvovirus genome) rather than for a specific virus protein (see, Cann, A.J., *Principles of Virology*, 2nd ed. Academic Press, 1997, pp. 139-140, Exhibit U). The mechanism of DNA replication of the AAV single-stranded genome is also very different from that of SV40, in which ITRs (which are unique to AAV) play a major role. This mechanism is very different from the mechanism of replication of the double-stranded circular SV40 DNA, which is a minichromosome. Importantly, AAV assembles by the introduction of genetic material into pre-formed capsids, a mechanism completely different from that of SV40. As explained by Berns and Linden (*BioEssays* 17:237-245, 1995, Exhibit V), "[t]he capsids are preformed and they interact with replicative intermediates to sequester single strands for encapsidation." Furthermore, "there is one report implicating Rep 52/40 [a unique AAV gene product] in this process" (page 242, bottom of left-hand column) In contrast, many studies have shown that SV40 assembles by gradual addition of the capsid proteins around the

chromatin, thus constructing the shells (reviewed by Bina, M., *Comments Mol. Cell. Biophys.* 4:55-62, 1986, Exhibit W).

In addition, U.S. Pat. No. 5,863,641 teaches the production of recombinant AAV capsid proteins, expressed from an adenoviral vector, and their use for *in vivo* encapsidation of AAV vector DNA into infectious viral capsids, and not the *in vitro* construction of SV40 pseudoviruses.

Furthermore, U.S. Pat. No. 5,863,641 describes (at section 5.3.2, columns 9-10) hypothetical methods for the *in vitro* assembly of AAV capsids, and *in vitro* encapsidation of components. The authors did not demonstrate that their hypothetical methods will work for AAV capsids. For *in vitro* assembly of SV40, those methods were investigated and were already demonstrated not to work, as explained below.

U.S. Pat. No. 5,863,541 suggests isolating AAV capsids produced *in vivo*, disassembling them by known techniques, and allowing them to re-assemble in the presence of the desired constituents. However, this method was tried for SV40 capsids in Christensen *et al.*, and again by Colomar (*J. Virol.* 67:2779-2788, 1993), and failed in both cases. The findings of Christensen *et al.* are described above. Colomar *et al.* attempted to package foreign DNA (polyoma virus DNA) in the disassembled SV40 particles. The titer of the reassembled particles decreased by 10,000-fold compared to the titer of the original virion preparation. Furthermore, Colomar *et al.* found that the particles containing foreign DNA had no biological activity, and were not infective. Therefore one of ordinary skill, applying the protocols described in U.S. Pat. No. 5,863,641 to SV40 would have failed to make Applicants' invention.

The Examiner also states that Carswell *et al.* (*J. Virol.* 60:1055-1061, 1986) teaches "the advantage of combining an SV40 agnoprotein with SV40 capsid proteins to facilitate the assembly of capsids." This reference shows that mutants which make no agnoprotein display abnormal perinuclear-nuclear localization of VP1, the major capsid protein (see Abstract), and that at least one function of the agnoprotein is to mediate the efficient localization of VP1 to the nuclear region, presumably so that the protein can participate in encapsidation (bottom of first column). However, this reference does not teach the making of constructs as described in the present invention, nor does it make any suggestion to do so. Rather, the goal of Carswell *et al.* is to elucidate the function of the SV40 agnoprotein, which was found to be the enhancement of the efficiency of localization of VP1 to the nuclear region. Nothing in Carswell *et al.* teaches or

suggests assembly of constructs containing exogenous nucleic acids similar to those described in Applicants' Claims 18, 29 or 35, upon which claims 23, 25-28, 31-34 and 36-38 depend.

The Office Action states that Oppenheim *et al.* (*Proc. Natl. Acad. Sci. USA* 83:6925-6929, 1986) teaches the advantage of combining an SV40 *ori* with SV40 capsid proteins to facilitate the assembly of capsids. Oppenheim *et al.* teach SV40 pseudovirions that contain the CAT reporter gene. Oppenheim *et al.* do not teach how to make or use the constructs, virions or pseudovirions of the present invention. The constructs of Oppenheim *et al.* do not contain an exogenous nucleic acid encoding a therpeutic gene product. Oppenheim *et al.* teach plasmids containing *ori,* or *ori* and *ses* that have little or no CAT activity (a measure of gene expression). In addition, Applicants have found that *ses* is required for *in vivo* packaging, but not *in vitro* packaging (Specification, page 30). Therefore, Oppenheim *et al.* do not render obvious constructs for expression of a protein product, as claimed in the instant application.

Forstova *et al.* and U.S. Pat. No. 5,863,541 make no mention of SV40 viruses, nor do they suggest that their methods would be useful in the manipulation of SV40. Christensen *et al.* were unable to produce infectious particles in any appreciable quantity which contained active DNA, and this reference demonstrates little else but a desire to produce such particles. Carswell *et al.* represents an academic study into the workings of SV40, and also offers no methods for production of infectious particles carrying exogenous nucleic acid, nor does it even suggest that this would be a desirabel thing to do. Oppenheim *et al.* fails to teach appreciable expression of exogenous nucleic acid.

The Federal Circuit has stated that "[o]bviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching[,] suggestion or incentive supporting the combination." (*In re Geiger*, 815 F.2d 686, 688, 2 U.S.P.Q.2d 1276, 1278 (Fed. Cir. 1987). To establish a *prima facie* case of obviousness, more is required. As stated by the court in *In re Geiger*, "[o]bviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching[,] suggestion or incentive supporting the combination." (815 F.2d 686, 688, 2 U.S.P.Q.2d 1276, 1278 (Fed. Cir. 1987). In *Geiger*, the PTO had failed to establish a *prima facie* case of obviousness, and the court went on to say that "[a]t best, in view of these disclosures, one skilled in the art might find it obvious to try various combinations of these known ... agents. However, that is not the standard of 35 U.S.C. §103." *Id.* at 1278.

Like *Geiger*, the references cited by the Examiner may be combined in a way to make it obvious to those in the field of SV40 virology to continue to try to make SV40 constructs which contain exogenous nucleic acid, and are safe for use in humans. But the Federal Circuit has long held that "obvious to try" does not constitute "obviousness." The court in *In re O'Farrell* (853 F.2d 894, 7 U.S.P.Q.2d 1673 (Fed. Cir. 1988)) made an excellent distinction between these two concepts. Judge Rich noted that "[a]ny invention that would in fact have been obvious under §103 would also have been, in a sense, obvious to try. The question is: when is an invention that was obvious to try nevertheless nonobvious?" (*Id.* at pages 1680-81). He went on to state that

> The admonition that 'obvious to try' is not the standard under § 103 has been directed mainly at two kinds of error. In some cases, what would have been 'obvious to try' would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful. *[4 case cites omitted]*. In others, what was 'obvious to try' was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it.

Id. at 1681.

Furthermore, Oppenheim *et al.* states that the SV40 *ori* region is essential, and that in order to effect efficient introduction of DNA into human bone marrow cells, their method "may be *suitable* . . . when a helper-free pseudoviral preparation becomes available" (page 6928, last paragraph). It is Applicants' belief that these statements constitute a teaching away from this area of endeavor until such time as "helper-free prudoviral preparation become available".

In *In re Gurley* (27 F.3d 551, 31 U.S.P.Q.2d 1130 (Fed. Cir. 1994)), the court stated that

> A reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path that was taken by the applicant . . .[or] if it suggests that the line of development flowing from the reference's disclosure is unlikely to be productive of the result sought by the applicant.

Id. at 553, 1131. Oppenheim *et al.* teaches away from Applicants' invention. Combining this reference with the others cited by the Examiner, offers no teaching, suggestion or incentive to lead one of ordinary skill in the proper direction to produce Applicants' claimed invention.

For these reasons, Applicants respectfully submit that the claimed invention is not obvious in view of the cited references, that one of ordinary skill would not have had a reasonable expectation of success in producing the claimed invention, and Applicants therefore respectfully request that the rejection on this basis be withdrawn and reconsidered.

Rejection in view of Forstova et al. or Christensen et al. in view of Carswell et al., Oppenheim et al. and U.S. Pat. No. 5,863,541, and further in view of Szczylik et al.

Claims 39 and 40 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Forstova *et al.* or Christensen *et al.*, in view of Carswell *et al.*, Oppenheim *et al.* and U.S. Pat. No. 5,863,541, and further in view of Szczylik *et al.* (*Science* 253:562-565, 1991). The Office Action states that Szczylik *et al.* teach an antisense oligonucleotide of *bcr/abl*, and that it would have been obvious to one or ordinary skill in the art to modify the methods of the cited references with the antisense oligonucleotide of Szczylik *et al.* to produce the instance invention.

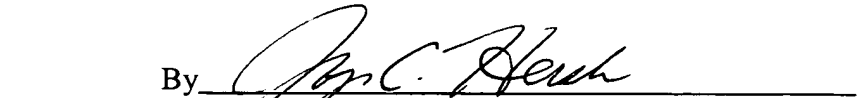
However, as stated above, Forstova *et al.*, Christensen *et al.*, Carswell *et al.*, Oppenheim *et al.* and U.S. Pat. No. 5,863,541 cannot be combined to produce the Applicants' invention because Forstova *et al.* and U.S. Pat No. 5,863,541 make no mention of SV40 viruses, Christensen *et al.* fails to obtain infectious particles in any appreciable frequency, and Carswell *et al.* represents merely a study on the function of SV40 agnoprotein. Oppenheim *et al.* teaches away from Applicants' invention. Because the combination of these references fails to produce Applicants' invention, the addition of Szczylik *et al.* also cannot, since it also fails to produce the constructs of Applicants' invention. Because none of the cited references, alone or in combination, produce Applicants' constructs, the rejection on 35 U.S.C. § 103 grounds should be withdrawn and reconsidered.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned at (781) 861-6240.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By 

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HUMAN GENE THERAPY 9:649–657 (March 20, 1998)
Mary Ann Liebert, Inc.

Efficient Transduction of Human Hematopoietic Cells with the Human Multidrug Resistance Gene 1 via SV40 Pseudovirions

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ABSTRACT

Transduction of *MDR1* may be of use in chemoprotection of normal bone marrow (BM) cells during treatment of malignancies, or as a selectable marker for the transfer of other genes into the BM, a critical target for the cure of many diseases. To that aim, the human multidrug resistance gene *MDR1* was cloned into an SV40 pseudoviral vector containing the SV40 origin of replication (*ori*) and encapsidation signal (*ses*), and the plasmid was encapsidated in COS cells as SV40/*MDR1* pseudovirions. Expression of the human *MDR1* gene was demonstrated in murine MEL cells infected with SV40/*MDR1* pseudovirions, using a monoclonal antibody (MPK16) specific for the human 170-kD P-glycoprotein. Functional P-glycoprotein was demonstrated by resistance to colchicine in NIH-3T3 cells infected with SV40/*MDR1* pseudovirions. Activity of P-glycoprotein was assayed by rhodamine-123 dye exclusion and fluorescence-activated cell sorter analysis (FACS) in various cell types including hematopoietic cells. Highly efficient gene transfer and expression was demonstrated in all murine and human cell types tested, including primary human BM cells. Using multiplicities of infection (moi) of 1–2, over 95% of cells were found to become *MDR1*$^+$. The percent of *MDR1*$^+$ cells was proportional to the moi. We conclude that the SV40 pseudoviral vector is efficient for gene transmission into human hematopoietic cells.

OVERVIEW SUMMARY

SV40 is an attractive potential vector with high-efficiency gene transfer into a wide variety of human tissues, including the bone marrow. Introducing the human *MDR1* gene into hematopoietic progenitor cells has been recently attempted to reduce the toxicity caused by anticancer drugs in patients undergoing chemotherapy. Here we demonstrate the use of SV40 pseudovirions to transfer a functional human *MDR1* gene into human bone marrow cells. The experiments demonstrated highly efficient gene transfer and significant expression. These studies, in parallel with the development of an *in vitro* packaging system for SV40 pseudovirions, suggest the feasibility of using the SV40/*MDR1* pseudovirion delivery system for chemotherapy protection. This system is also potentially valuable for delivering other therapeutic genes into hematopoietic cells, a critical target for the treatment of many diseases.

INTRODUCTION

THE MAJORITY OF GENE TRANSFER STUDIES and clinical trials have been performed using retroviral vectors. However, many considerations have led to the search for alternate vectors, including the difficulty of gene transfer into stem cells, suboptimal engraftment of retrovirally transduced cells, as well as safety issues.

SV40 is a papovavirus whose genome is a circular double-stranded DNA 5.2 kb in size. We have chosen this vector for its wide, perhaps unlimited, host range and the extensive knowledge regarding its biology and molecular biology. Importantly, SV40 is probably harmless to humans. It was originally discovered as a contaminant in polio vaccines in the United States. Thus, millions of individuals of all ages were inadvertently infected with SV40 in the 1950s. From random testing, it is known that many people carry antibodies to SV40 but the virus has not been associated with human disease (Geissler, 1990).

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Several years ago, we began to develop SV40 pseudovirions for the purpose of gene transfer into hematopoietic cells (Oppenheim *et al.*, 1986). The pseudovirions can transmit plasmids that carry over 90–95% of human DNA, and they contain only a few hundred base pairs of nonhuman origin, with a cloning capacity of ~5.3 kb (Dalyot and Oppenheim, 1989; Oppenheim *et al.*, 1992). They transmit DNA at high efficiency into a variety of cells, including human hematopoietic cells. In early experiments, over 30% of fresh human bone marrow (BM) cells and 42% of K562 human erythroleukemia cells became infected (Oppenheim *et al.*, 1986, 1987), and the expression of a reporter gene (*cat*) was high. For clinical applications, we have recently begun developing *in vitro* packaging of pseudovirions. We have demonstrated that pseudovirions may be prepared in the test tube, under controlled conditions, using plasmid DNA purified from *Escherichia coli* and recombinant capsid proteins produced in insect cells (Sandalon *et al.*, 1997). Furthermore, viral packaging *in vitro* was found to be flexible, allowing packaging of significantly larger plasmids (at least 7.5 kb) with minimal SV40 sequences (150 bp). Importantly, this approach combines the efficiency of gene delivery characteristic of viral vectors with the safety and flexibility of nonviral delivery systems. Here we have tested the feasibility of using SV40 pseudovirions for the transfer of the 3.8 kb gene for multidrug resistance, *MDR*1.

*MDR*1 (Gottesman and Hrycyna, 1995) encodes a 170-kD plasma membrane glycoprotein (P-glycoprotein), which confers energy-dependent resistance to a number of naturally occurring, structurally unrelated types of chemotherapeutic agents, including anthracyclines, vinca alkaloids, epipodophyllotoxins, taxol, and actinomycin D (Gottesman and Hrycyna, 1995). Recently, it was recognized that a small population of normal human BM cells, which are probably stem cells, also express *MDR*1 (Chaudhary and Roninson, 1991). However, the overall expression and/or the total number of *MDR*1-expressing cells is necessarily low, because this tissue is highly chemosensitive and recent studies have shown that functional P-glycoprotein is not present on normal granulocytes (Klimecki *et al.*, 1994). This provides the rationale for *MDR*1 gene transfer as a strategy for chemoprotection of BM against cytotoxic chemotherapy.

Many malignancies respond partially to chemotherapy but cannot be eradicated with conventional doses. If the BM is free of disease, it can be removed from the patient and reinfused after high-dose chemotherapy (autografting). However, following chemotherapy, during the time required for reconstitution of the autograft (a period of several weeks in heavily treated patients), serious complications can develop due to BM suppression.

Recent experiments suggest that expression of an exogenous *MDR*1 gene may prevent chemotherapy-induced BM suppression. Transgenic mice expressing the human *MDR*1 gene showed long-term resistance to chemotherapy-induced neutropenia (Mikisch *et al.*, 1992). In addition, mice transduced with an *MDR*1 gene showed expression of the gene in granulocytes and in BM cells (Podda *et al.*, 1992; Sorrentino *et al.*, 1992) and resistance to chemotherapy-induced neutropenia (Hanania and Deisseroth, 1994; Hanania *et al.*, 1995; Aksentijevich *et al.*, 1996). Two groups have reported successful transduction of human $CD34^+$ cells with an *MDR*1 cDNA (Hanania *et al.*, 1994; Ward *et al.*, 1994), suggesting the feasibility of this approach for human therapy. These experiments were performed using retroviral vectors.

However, retrovirus-mediated transfer of *MDR*1 has several limitations. First, although these vectors can be used to introduce *MDR*1 into murine stem cells (Licht *et al.*, 1995), they are inefficient with nondividing cells such as the human BM stem cell (Hanania *et al.*, 1996). This necessitates exposing the patient or his BM cells *ex vivo* to prior chemotherapy and cytokines to induce the cells to cycle (Licht *et al.*, 1995). The need for the development of alternate gene delivery systems led us to test the SV40 vector, which is suitable for nondividing cells, for efficiency of *MDR*1 gene transduction.

MATERIALS AND METHODS

Cell lines and cultures

COS (Gluzman, 1981) and CMT4 cells (Gerard and Gluzman, 1985) are African green monkey kidney cells that harbor the viral genes for T antigen, K562 is a human erythroleukemia cell line. NIH-3T3 are murine fibroblasts. $P388^S$ (Inaba *et al.*, 1979) is a chemosensitive mouse lymphoblastic leukemia line, widely used in studies on sensitivity to chemotherapy. MEL is a mouse erythroleukemia cell line, transformed by Friend virus. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS; COS, CMT4, NIH-3T3) or RPMI with 10% FCS (K562). $P388^S$ was maintained in RPMI with 10% FCS and 10 μM β-mercaptoethanol. Human BM cells, obtained from healthy volunteer donors for BM transplantation, were separated on Ficoll-Hypaque gradients and maintained in RPMI with 10% FCS until infection (24–48 hr). Use of volunteer donor marrow was performed in accordance with the guidelines of the Hadassah University Hospital Helsinki Committee.

Vector construction

pSO41 carries the SV40 early promoter and early polyadenylation signal and a cloning site located between these two elements (Fig. 1A). The human *MDR*1 cDNA, derived from p*MDR*2000 (Ueda *et al.*, 1987a), was inserted between the *Xba* I and *Hind* III sites by multiple cloning steps (Dagan, 1994). Several plasmid constructs were prepared and tested for *MDR*1 expression in CMT4. Plasmid pSM1 (Fig. 1B), containing a 4,361-bp *MDR*1 cDNA fragment (coordinates 286–4,646, GenBank accession number M14758), was chosen for further experiments. Its structure was verified by sequence analysis of the cloning junction (Dagan, 1994).

Transcription analysis of MDR1

Total cellular RNA was extracted using TriReagent (Molecular Research Center, Inc., Cincinnati, OH). Reverse transcriptase-polymerase chain reaction (RT-PCR) (Kawasaki *et al.*, 1988) was performed with Moloney murine leukemia virus reverse transcriptase (BRL) using approximately 1 μg of RNA. Primer D8 was used for the RT reaction. One-fifth of the product was amplified by PCR in a total volume of 50 μl, using 18 ng of each primer, for 35 cycles. The amplification cycle was: 1.5 min at 92°C, 1 min at 52°C, and 1 min at 72°C. Then, 10-

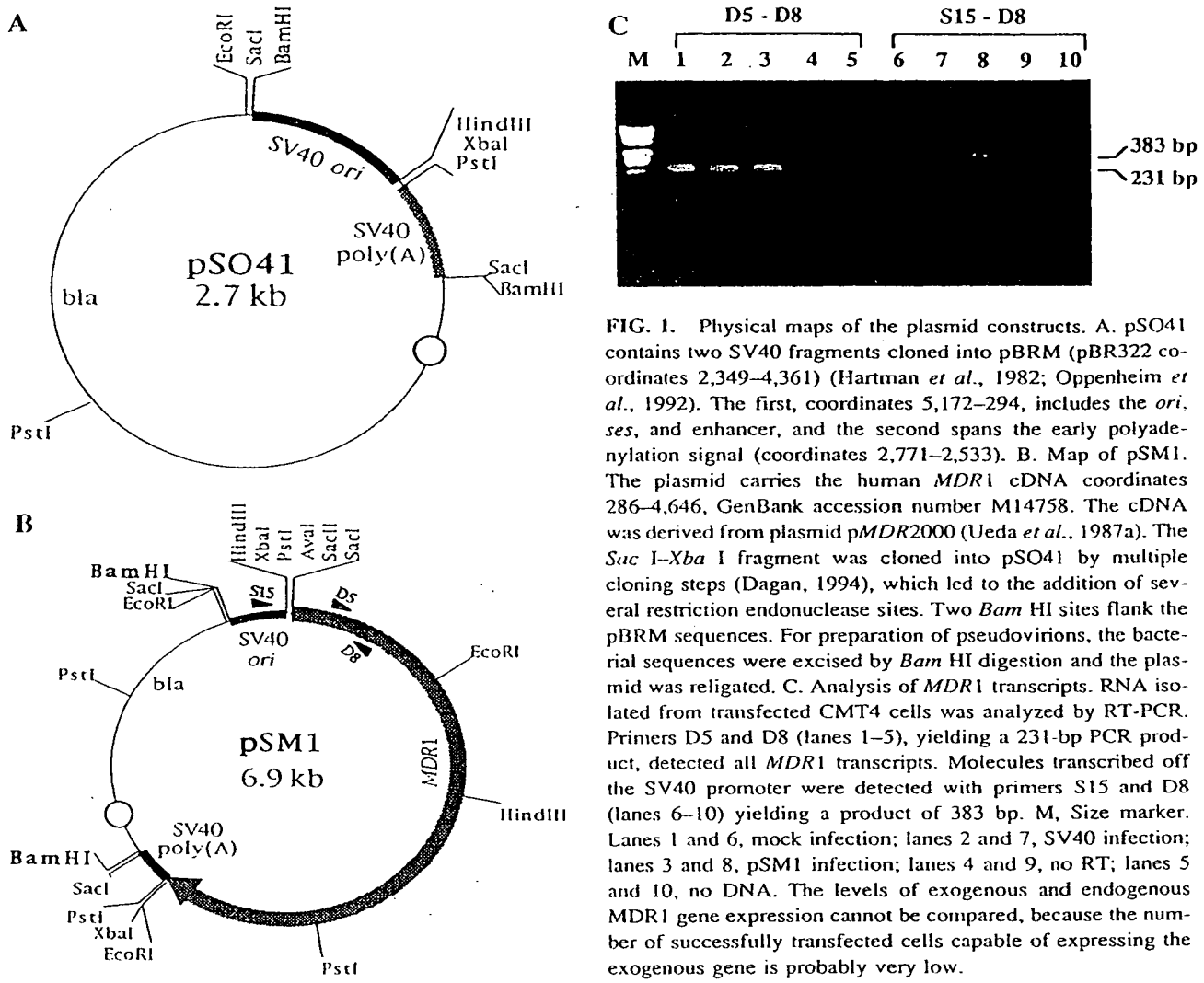

FIG. 1. Physical maps of the plasmid constructs. A. pSO41 contains two SV40 fragments cloned into pBRM (pBR322 coordinates 2,349–4,361) (Hartman *et al.*, 1982; Oppenheim *et al.*, 1992). The first, coordinates 5,172–294, includes the *ori*, *ses*, and enhancer, and the second spans the early polyadenylation signal (coordinates 2,771–2,533). B. Map of pSM1. The plasmid carries the human *MDR1* cDNA coordinates 286–4,646, GenBank accession number M14758. The cDNA was derived from plasmid p*MDR*2000 (Ueda *et al.*, 1987a). The *Sac* I–*Xba* I fragment was cloned into pSO41 by multiple cloning steps (Dagan, 1994), which led to the addition of several restriction endonuclease sites. Two *Bam* HI sites flank the pBRM sequences. For preparation of pseudovirions, the bacterial sequences were excised by *Bam* HI digestion and the plasmid was religated. C. Analysis of *MDR1* transcripts. RNA isolated from transfected CMT4 cells was analyzed by RT-PCR. Primers D5 and D8 (lanes 1–5), yielding a 231-bp PCR product, detected all *MDR1* transcripts. Molecules transcribed off the SV40 promoter were detected with primers S15 and D8 (lanes 6–10) yielding a product of 383 bp. M, Size marker. Lanes 1 and 6, mock infection; lanes 2 and 7, SV40 infection; lanes 3 and 8, pSM1 infection; lanes 4 and 9, no RT; lanes 5 and 10, no DNA. The levels of exogenous and endogenous MDR1 gene expression cannot be compared, because the number of successfully transfected cells capable of expressing the exogenous gene is probably very low.

μl samples of the PCR reaction were analyzed on 2% NuSieve agarose gels. The PCR primers were: S15, 5′-AGAAGTAGTGAGGAGGCT-3′ (SV40 coordinates, 5,220–5,203); D5, 5′-CCACTAAAGTCGGAGTATCTTC-3′ (*MDR1* coordinates 355–375); D8, 5′-CCACCACCATATACAACTTGTC-3′ (*MDR1* coordinates 584–563).

Preparation of pseudoviral stocks

Prior to packaging, bacterial sequences were excised from pSM1 by *Bam* HI digestion and the DNA was religated at low DNA concentration (5 μg/ml), as previously described for β-globin constructs (Dalyot and Oppenheim, 1989). Viral stocks were prepared by transfection of COS cells using the DEAE-dextran method (McCutchan and Pagano, 1968), with 3 μg of pSM1 DNA, and 1.5 μg of SV40 wild-type (wt) DNA (BRL) as a helper, per 75-cm^2 culture flask at 60–70% confluence. After 5 days of incubation, viral stocks were harvested by repeated freeze–thaw followed by chloroform treatment. For the last 2 days, the cultures contained only 6 ml of media per 75-cm^2 flask, to increase the viral titer. Viral stocks were titered as infective centers on CMT4 cells. Some viral stocks were concentrated using Aquacide (Calbiochem-Novabiochem, La Jolla, CA) or Polyethylene glycol 6000 (Merck). The viral stocks were placed in dialysis tubing (12,000–14,000 Daltons, Medicell Ltd., Liverpool, UK), which was submerged in the granular reagent for 20–60 min, until the volume was reduced two- to five-fold. The concentrated stocks were retitered.

Pseudoviral titration

Pseudoviral stocks were titered as infective centers on CMT4 monolayers. The SV40 T antigen in CMT4 is expressed under the inducible metallothionein promoter (Gerard and Gluzman, 1985). Subconfluent monolayers were infected at several dilutions, according to the infection protocol described below. Fol-

lowing infection, T antigen expression was induced by the addition of 0.1 m*M* $ZnCl_2$ and 1 μM $CdSO_4$, to allow propagation of the SV40/*MDR*1 DNA in the infected CMT4 cells. Infective centers, identified and scored by *in situ* hybridization with an *MDR*1-specific probe, were used to calculate the titer.

SV40 pseudovirion infection

Viral stock was added to monolayers, and allowed to adsorb for 2 hr at 37°C with occasional gentle mixing. In parallel, cells were infected with wt SV40 at a concentration corresponding to that present in the pseudovirion mixture (10-fold greater than the *MDR*1 pseudovirions). Another control was mock infected, using a freeze-thaw lysate prepared from untransfected COS cells. The infected and control cells were then washed, and fresh medium was added. Hematopoietic cells, including Ficoll-separated mononuclear bone marrow preparations, were infected in round-bottomed culture tubes, with occasional gentle agitation during the 2-hr adsorption period. The infected cells were centrifuged (<1,000 rpm for 3 min), supernatants were removed, and fresh medium was added.

Rhodamine-123 dye exclusion assay

The cells were incubated with 150 ng/ml rhodamine-123 (Sigma) for 15 min at 37°C, then washed and allowed to efflux the dye for 2 hr at 37°C. Cells were kept at 4°C until analyzed by fluorescence-activated cell sorting (FACS), on the same day. Immediately prior to sorting, the cells were counterstained with propidium iodide.

Analysis of human P170 by specific antibodies

The monoclonal antibody MRK16 (Hamada and Tsuruo, 1986), a gift of Hoechst Japan, Ltd (Kawagoe City, Saitama, Japan) is specific for human P-glycoprotein and does not cross-react with murine P-glycoprotein. A total of 5×10^5 MEL cells were infected with pSM1 pseudovirions and analyzed. The cells were suspended in 50 μl of phosphate-buffered saline (PBS), and 1% bovine serum albumin (BSA) at 4°C with 1 μg MRK16 antibody, for 30 min. After washing with PBS and 1% BSA, the cells were stained with an fluoroisothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) (4°C for 30 min), washed, and analyzed by FACS.

Determination of drug resistance

NIH-3T3 cells were infected, when ~60% confluent, with mock lysates, SV40 wt, and SV40/*MDR*1 pseudovirions. Selection was performed at 40, 50, and 60 ng/ml of colchicine. Cells were observed daily for signs of colchicine toxicity and for the appearance of drug-resistant colonies. Media with or without colchicine was changed twice weekly.

RESULTS

Construction and expression of MDR*1-containing pseudovirions*

The human *MDR*1 cDNA was cloned into the SV40-based vector pSO41 (Fig. 1A). The vector includes the SV40 sequences necessary for packaging, comprising the viral *ori* (origin of replication), *ses* (SV40 encapsidation signal; Oppenheim *et al.*, 1992, the SV40 enhancer, and the SV40 early polyadenylation signal (Fig. 1B). The *MDR*1 fragment includes 137 bp upstream of the ATG signal, excluding the downstream promoter and regulatory elements (Ueda *et al.*, 1987b).

To test whether the cloned *MDR*1 gene is expressed off the SV40 early promoter, we performed RT-PCR analysis on RNA extracted from CMT4 cells transfected with pSM1 DNA. Two pairs of primers were used. One was designed to detect transcripts that start within the SV40 sequences, by amplifying across the SV40-*MDR*1 junction, with primers S15 and D8 (Fig. 1B). The sequence amplified starts 38 nucleotides downstream from the TATA box of the early promoter, coordinates 5,220–5,203, and ends in *MDR*1 sequences (383 nucleotides). The second primer pair (D5 and D8), which amplifies 231 nucleotides within the *MDR*1 cDNA, detects all *MDR*1 transcripts including those of the endogenous gene. As seen in Fig. 1C, *MDR*1 transcripts were detected with primers D5 and D8 in all cells, including mock and SV40 transfected, indicating expression of the endogenous gene. Transcripts that include SV40 sequences (primers S15 and D8) were detected only in the pSM1-transfected cells. These results indicate that the SV40 early promoter drives expression of *MDR*1 in pSM1.

pSM1 pseudovirions were prepared by co-transfecting COS cells with the plasmid together with wt SV40 DNA as a helper. The cells and the helper supply the viral large T antigen, allowing plasmid DNA replication. The helper provides the late viral gene products, VP1, VP2, and VP3, required for encapsidation (Oppenheim and Peleg, 1989). The pSM1 pseudoviral titer was generally around $4–5 \times 10^4$ as determined by infective centers in CMT4 cells, using *in situ* hybridization with an *MDR*1-specific probe. Pseudoviral stocks were concentrated to a titer of approximately $2–5 \times 10^5$/ml of pSM1. The SV40 present in the pseudovirion mixture was usually 10-fold higher. The numbers represent a minimal titer because the assay requires that the plasmid replicate extensively in the infected cells to score as positive. The experiments described below suggest that the true titer of pSM1 pseudovirions was two- to three-fold higher.

Expression of the human MDR*1 gene in murine hemopoietic cells*

Transduction of the human *MDR*1 gene was tested in mouse hematopoietic cells using a human-specific monoclonal antibody, MRK16. MEL cells were infected at an moi of 0.3, stained with MRK16, and subjected to FACS analysis. Mock and SV40 infections served as controls. It can be seen (Fig. 2) that at least 65–70% of the cells had the human P-glycoprotein identified on their surface, whereas control SV40-infected cells and the mock-infected cells were negative (Fig. 2). The discrepancy between the moi and the percentage of P-glycoprotein-positive cells is most likely due to an underestimation of the titer. The results demonstrate efficient transmission and expression of the exogenous human gene in a mouse hematopoietic cell line.

Production of functional P-glycoprotein in pSM1 pseudovirion-transduced cells

Functional P-glycoprotein may be analyzed by rhodamine-123 dye exclusion because it effluxes such fluorescent dyes

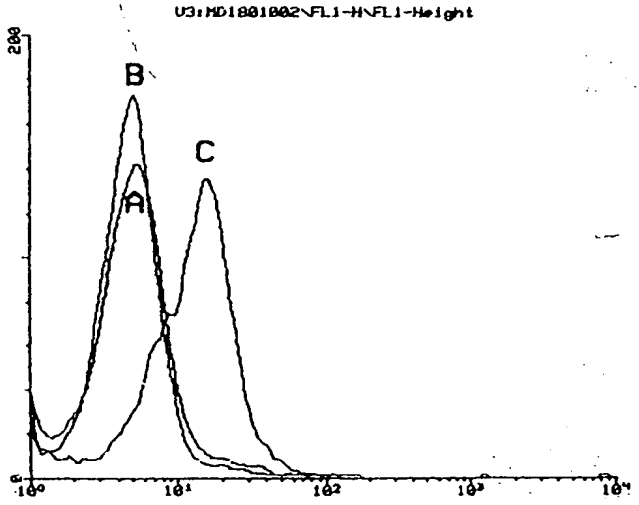

FIG. 2. Human P-glycoprotein expression in murine cells. MEL cells were infected with pSM1 pseudovirions and stained with MRK16, an antibody specific for human P-glycoprotein, which does not cross-react with murine P-glycoprotein. Mock-infected cells served as a control. Line A represents mock infection; line B is SV40 infection; line C is pSM1 pseudovirion-infected cells. The *x* axis represents MRK16 fluorescence on a logarithmic scale, and the *y* axis, relative cell number.

from cells (Chaudhary and Roninson, 1991). The human erythroleukemia cell line K562 was infected with pSM1 pseudovirions at various moi. When stained with rhodamine-123, mock-infected and SV40-infected cells appeared as a single superimposable population of bright cells, indicating no P-glycoprotein activity (Fig. 3A, left panel). In the pSM1 pseudovirion infections (Fig. 3A, right panel, curves A–D), a portion of the cells became dull-staining, indicating the presence of a functional *MDR1* gene product in those cells. As the moi increases from 0.2 to 1.0 (Fig. 3A, panel on right, curves A–D), the proportion of dull cells increases (left-hand portion of the curves) and that of brightly stained cells decreases (right-hand portion of curves). Quantification of the data show that the percent of "dull" cells was proportional to the moi (Fig. 3B). The results suggest highly efficient gene transduction. Infections were repeated 20 times using five different batches of pseudovirions, and the results reproducibly showed efficient *MDR1* transduction (in repeated experiments, between 73% and 97% of infected cells at moi of 1.0 were $MDR1^+$). Efficient *MDR1* transduction was also seen using the monoclonal antibody MRK16 (not shown).

Similar studies were also performed in two drug-sensitive strains of murine cell lines, MEL and $P388^S$. In these experiments, the percent of $MDR1^+$ cells was also proportional to the moi, with approximately 90% of cells $MDR1^+$ at an moi of 1. The infections were repeated several times using four different batches of pseudovirions, with consistently reproducible results.

pSM1 pseudovirions confer drug resistance in transduced murine fibroblasts

NIH-3T3 cells, infected with pSM1 pseudovirions at an moi of 0.06, were subjected to selection in media containing colchicine. NIH-3T3 cells have been shown to have a low level of endogenous *MDR1* expression, quantitated as 359 P-glycoprotein molecules on average per cell (Broxterman *et al.*, 1997). A marked difference was observed in the appearance of the cells in the pSM1 pseudovirion-infected cultures versus the mock-infected, beginning 3 days after selection in 50 ng/ml colchicine (Fig. 4). After 28 days of selection in 50 ng/ml colchicine, 64 drug-resistant colonies were scored in the pSM1 pseudovirion-infected cultures compared to three colonies in the mock-infected control cultures. SV40 infection did not lead to colchicine-resistant colonies.

Transduction of primary human BM cells

Normal human BM cells (obtained from healthy volunteer donors for BM transplantation with approval of the Hadassah University Hospital Helsinki Committee) were infected with pSM1 pseudovirions under the same conditions. As seen in Fig. 5, at baseline, human BM has more cells able to efflux rhodamine, as compared to cell lines. Nonetheless, it can be seen that although there is a higher number of dull-staining cells prior to infection (Fig. 5A), their number increases greatly upon transduction with pSM1 pseudovirions at moi 1.0 (Fig. 5C), reaching 97% rhodamine "dull". The number of "dull" staining cells did not increase following SV40 infection (Fig. 5B). The experiment was repeated using BM cells obtained from 7 individuals, with consistent results (87–97% $MDR1^+$). Furthermore, the proportion of $MDR1^+$ cells was found to increase linearly with moi (not shown). Further support for efficient transduction of primary human bone marrow cells was obtained using analysis with the monoclonal antibody MRK16 (not shown).

DISCUSSION

We have utilized the SV40-based pseudoviral system for transfer of the human *MDR1* gene. We have demonstrated highly efficient gene transfer into all types of cells tested, including fresh unmanipulated primary human BM cells. *MDR1* gene expression was demonstrated at the RNA and protein levels. The SV40/*MDR1* vector conferred drug resistance in transduced murine cells. Furthermore, the P-glycoprotein was found to be active as measured by rhodamine efflux in different types of transduced cells. Because SV40 has a wide host range, and it infects nondividing cells, we anticipate that it is capable of transducing BM stem cells. Indeed we found almost 100% transduction in the absence of cytokine treatment. Further experiments are required to verify whether stem cells are being transduced.

Retroviral vectors are currently most commonly used for *MDR1* gene delivery. Transduction efficiency, in cells exposed to multiple growth factors, ranged from 14% in transplanted mice (Podda *et al.*, 1992) to 62% of murine BM cells (Licht *et al.*, 1995) and from 20% in human CD34-selected BM cells (Hanania *et al.*, 1995) to 60–70% of cultured progenitors derived from human BM cells (Ward *et al.*, 1994). Liposomes have also been used for *MDR1* gene transfer into murine hematopoietic cell lines with retroviral (Aksentijevich *et al.*, 1996) and AAV-based vectors (Baudard *et al.*, 1996). Effi-

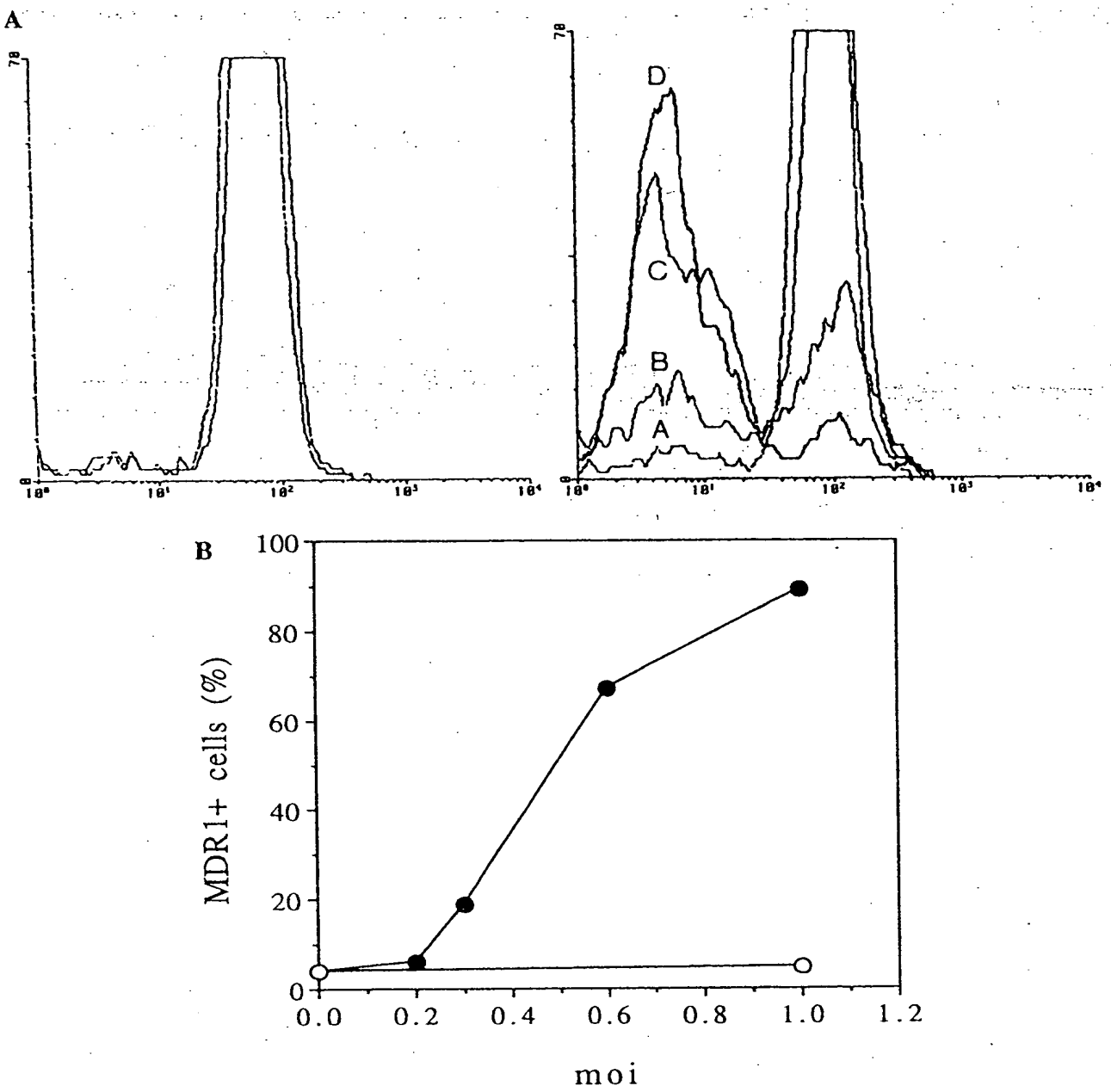

FIG. 3. *MDR*1 analysis using rhodamine-123 staining. A. K562 cells were infected with pSM1 pseudovirions at different multiplicities. Cells were analyzed using rhodamine-123 staining 3 days post infection. The *x* axis represents rhodamine-123 fluorescence on a logarithmic scale and the *y* axis, relative cell number. Panel at left: Mock infection superimposed on SV40 infection (at moi 10, the concentration is equal to that present in the virion mix with a pSM1 moi of 1.0); panel at right, A–D, pSM1 pseudovirion-infected cells at moi 0.2 (A), 0.3 (B), 0.6 (C), 1.0 (D). The curves A–D appear in ascending order in the lefthand portion, representing increasing dull-staining cells (*MDR*1$^+$) with increasing moi. On the right (bright staining region) the order of the curves is reversed. B. Quantification of the data. The percent of *MDR*1$^+$ cells (rhodamine dull) was determined by gating. ( ) Cells transduced with pSM1 pseudovirions; (O) infected with SV40 at a multiplicity 10× higher than shown for pSM1 pseudovirions on the *x* axis.

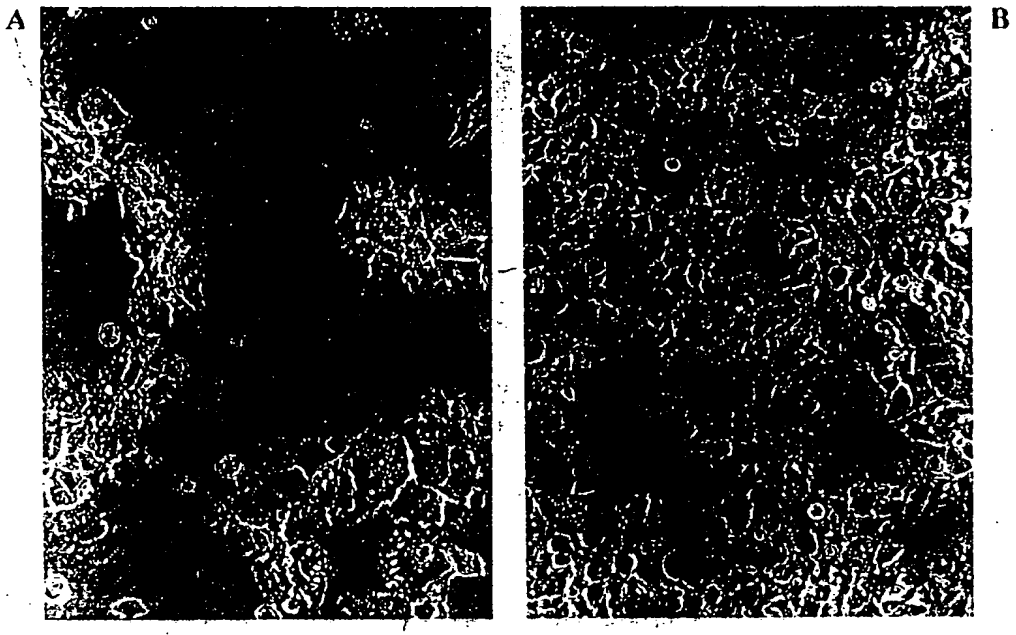

FIG. 4. Drug resistance in fibroblasts. NIH-3T3 cells were infected with pSM1 pseudovirions and subjected to selection in 50 ng/ml colchicine. Cells were observed daily for signs of toxicity. Prior to the appearance of isolated colonies, there was a marked difference in the confluence and appearance of the transduced cells compared to mock-infected cells, suggesting resistance to colchicine. A. Mock-infected cells. B. pSM1 pseudovirion-transduced cells, both photographed on day 13.

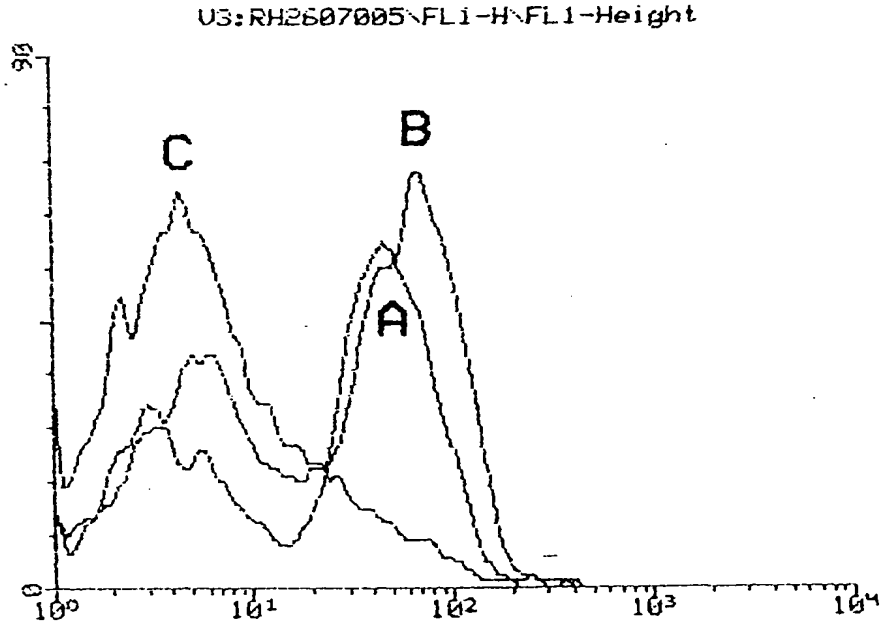

FIG. 5. *MDR1* transduction of human BM cells. A total of 1.5×10^4 cells were infected with pSM1 pseudovirions at an moi of 1 and analyzed by FACS using rhodamine-123 exclusion 24 hr post infection. SV40 and mock infection (as described for Fig. 3) served as controls. The *x* and *y* axes are as described for Fig. 3. Line A is mock infection, line B SV40 infection, moi as in Fig. 3, and line C pSM1 pseudovirion infection, moi 1.0. Analysis by gating demonstrated that 33%, 36%, and 87% of the cells, in A, B, and C, respectively, are rhodamine-dull.

ciency of transfection was 30% of unselected and 66% of preselected murine BM cells, as demonstrated by drug resistance in an *in vitro* CFU-mix assay. Somewhat lower transfection efficiencies were seen in an *in vivo* BM transplant model. There are no reports of using liposomes for *MDR*1 transfection of human hematopoietic cells.

For the purpose of chemoprotection, several weeks of *MDR*1 expression may be sufficient. In our preliminary studies, pSM1 pseudovirion infected MEL cells showed persistence of a rhodamine-dull population for the duration of the experiment, which was 6 weeks. Recent experiments have demonstrated that SV40 vectors confer stable gene expression in murine hematopoietic cells for over 100 days (Strayer and Milano, 1996). To avoid undesirable long-term expression, the use of suicide vectors (Sugimoto *et al.*, 1995) would be possible.

The presence of the SV40 helper in the pseudoviral stocks makes them inappropriate for human use. Recently, the possibility of preparing helper-free pseudovirions in the test tube, using DNA purified from *Escherichia coli* and capsid proteins produced in insect cells, has been demonstrated (Sandalon *et al.*, 1997). This *in vitro* packaging procedure will provide safe pseudoviral stocks suitable for medical applications, because it is based on the use of purified reagents that interact under aseptic conditions. The procedure is presently undergoing development to increase its efficiency. The present work, which demonstrates the high efficiency of SV40 pseudovirions in the transfer of functional genes into the human bone marrow, suggests that this gene delivery system should prove to be useful for future human use.

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Expression of β-Globin in Primary Erythroid Progenitors of β-Thalassemia Patients Using an SV40-Based Gene Delivery System

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ABSTRACT

SV40-based vectors are very efficient in gene delivery into human hematopoietic cells. In the present work, we investigated the expression of constructs carrying the human β-globin gene that were delivered as β-globin pseudovirions. Expression studies were performed by RNA analysis of primary human erythroid progenitors cultivated from peripheral blood of β^0-thalassemia patients who are unable to produce normal β-globin RNA. This erythroid culture system recapitulates in vitro the process of growth, differentiation, and maturation of authentic erythroid precursors. The progenitors were induced to differentiate by the addition of erythropoietin (EPO). Five days later, the cells were infected with pseudovirions containing the normal β-globin gene, and RNA was harvested on day 8. The results showed significant levels of normal β-globin gene mRNA. A small DNA fragment derived from the 5′-region of the HSII element of the human β-globin locus control region (LCR) enhanced expression of the linked β-globin gene 20–30-fold. Normal β-globin mRNA expression was in direct correlation to the multiplicity of infection. These studies suggest the potential feasibility of using the β-globin delivery system for gene therapy of β-thalassemia.

INTRODUCTION

β-THALASSEMIA IS A WORLDWIDE HEALTH PROBLEM and is one of the most common inherited disorders of humans. The patients have lifelong, transfusion-dependent anemia, with supportive care being both painful and extremely costly. The disease is curable by allogeneic BMT, a high-risk procedure requiring an HLA-matched related donor. Because autologous BMT (ABMT) has far fewer complications, it is attractive to consider the therapeutic potential of autologous transplantation of BM cured by gene therapy. Such transplantations could be models for ex vivo gene therapy of BM diseases.

The β-globin gene was among the first human genes cloned, and, thus, β-thalassemia was among the first target diseases for ex vivo gene therapy (1–3). However, major difficulties have been encountered, hindering the development of gene therapy for this disease.

The first problem is transduction of the human BM cell. Despite the success in transducing murine stem cells (generally using retroviral vectors) (1,4–6), the human hematopoietic stem cell has been extremely difficult to transduce. Retroviral vectors are inefficient with nondividing cells, such as the human BM stem cell (2,7). Exposure of the patient or the patient's BM cells ex vivo to prior chemotherapy and cytokines to induce the cells to cycle (7,8) causes some of the transduced cells to undergo differentiation before reinfusion into the patient, and genuine stem cells are not transduced (7). Adeno-associated virus (AAV)-based vectors require extremely high multiplicities of infection (MOI), from 10^3 to 5×10^7 particles per cell (9,10). Furthermore, retroviral and AAV sequences can dramatically silence transgene expression (11). Clearly, alternate viral vectors capable of infecting BM cells must be developed (12).

Another problem is that successful therapy of tha-

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lassemia requires high-level regulated expression of the β-globin gene, which is expressed at very high levels during a short time window in the developing erythrocyte. Appropriate regulation may be achieved by including regulatory elements derived from the β-globin locus control region (LCR) (13). However, these potent regulatory elements were found to cause extensive rearrangements with various viral vectors (see, for example, 14,15).

Simian virus 40 (SV40) is an attractive potential vector for gene therapy. Its biology and molecular biology have been studied extensively. The virus is easily handled and readily monitored, and high-titer stocks can be prepared that can be purified. The viral segment required for efficient packaging of SV40 pseudovirions is only 200 bp, encompassing the viral origin of replication (*ori*) and packaging signal (*ses*) (16). Thus, minimal interference with gene regulation is expected. Importantly, wild-type SV40 is probably harmless to humans. It was originally discovered as a contaminant in polio vaccines in the United States. Millions of people have thus been inadvertently infected with SV40 since the 1950s. Epidemiologic studies have clearly shown no adverse effects on the health of those immunized with the lots of vaccine containing SV40 (17–20).

Plasmids carrying the *ori-ses* region can be packaged in COS cells (see Materials and Methods) as SV40 pseudovirions. The SV40 capsid proteins are supplied in *trans* by helper SV40, cotransfected into the COS cells together with the plasmid of interest. This vector achieves very efficient gene delivery into K562 and human BM cells (21,22). In our recent studies, almost all (87%–97%) primary human BM cells infected at a low MOI, 1–2, with pseudovirions carrying the *MDR*1 gene became *MDR*1$^+$ (22). As SV40 also infects nondividing cells, it is expected to be capable of gene delivery into human hemopoietic stem cells.

A major difficulty in studying expression of β-globin constructs is the lack of an appropriate human cell line expressing the adult human β-globin gene. We have used primary erythroid progenitor cultures as an in vitro model system for studying β-globin transduction. These cell cultures recapitulate growth, differentiation, and maturation of erythroid progenitors in vitro. Cultures can be cultivated from peripheral blood of both normal and thalassemia patients (23), obviating the need for withdrawing BM by invasive procedures. In cultures obtained from patients with β^0-thalassemia, β-globin mRNA levels are very low, as in β-thalassemia patients, because of instability of the mutated β-globin message (24). Nevertheless, the cells maintain the potential to express their globin genes following erythropoietin (EPO) induction. Moreover, the mutations in the endogenous β-globin alleles allow distinction between RNA produced by the endogenous genes and that produced by the exogenous normal gene. These authentic primary hematopoietic progenitors were our target cells for β-globin gene transduction.

MATERIALS AND METHODS

Patients

Peripheral blood was drawn from patients with thalassemia major immediately before routine blood transfusion. All the patients had transfusion-dependent β^0-thalassemia major and thus were unable to produce normal β-globin RNA. Informed consent was obtained in accordance with Helsinki Committee guidelines, and approximately 10 ml of blood was cultured.

Packaging of β-globin pseudovirions

Before packaging, the bacterial sequences were removed from the plasmids (Fig. 1) by digestion with SacI and religation at low concentration (1 μg DNA in a 200 μl reaction). The DNA was treated with phenol-chloroform and was ethanol precipitated. Pseudovirions were prepared as previously described (21) in COS (monkey kidney) cells, which constitutively express SV40 T antigen. Cells, logarithmically grown in DMEM containing 10% FBS, were cotransfected with plasmid (3 μg DNA per 75 cm^2 culture) and SV40 DNA (150 ng) by the DEAE-dextran procedure. After 2 days, a portion of the medium was removed, and incubation was continued for 3 additional days in a volume of 6 ml medium per culture. Viral stocks were harvested by repeated freeze-thawing and chloroform treatment. For further concentration, the viral stock was centrifuged through a Centricon column. Viral titers were determined by in situ hybridization (22) using a β-globin-specific probe.

Cell culture system for β-globin expression studies

The experimental system is based on erythroid progenitors (burst-forming units–erythrocyte [BFU-E]) present in human peripheral blood, cultured in two phases (27). In the first phase, the BFU-E proliferate and differentiate into the more mature progenitors, colony-forming units–erythrocyte (CFU-E). The cells are then washed with saline and replated in the presence of EPO. During the second phase, the CFU-E further proliferate and mature into hemoglobin-containing cells (Fig. 2).

Total mononuclear cells (MNC), isolated by Ficoll-Hypaque from 10 ml peripheral blood of thalassemia patients, were cultured as described by Fibach et al. (27,28). In the second phase, 4–5 days after the addition of EPO, the erythroid cells were separated on a Percoll gradient. Most of the isolated erythroid cells were large, immature, nonhemoglobinized cells. Aliquots of 2×10^5 cells were infected at an MOI of 0.02–2.0. The number of cells harvested 3 days later varied because of the different proliferation rates of the individual cultures. Viable cells and

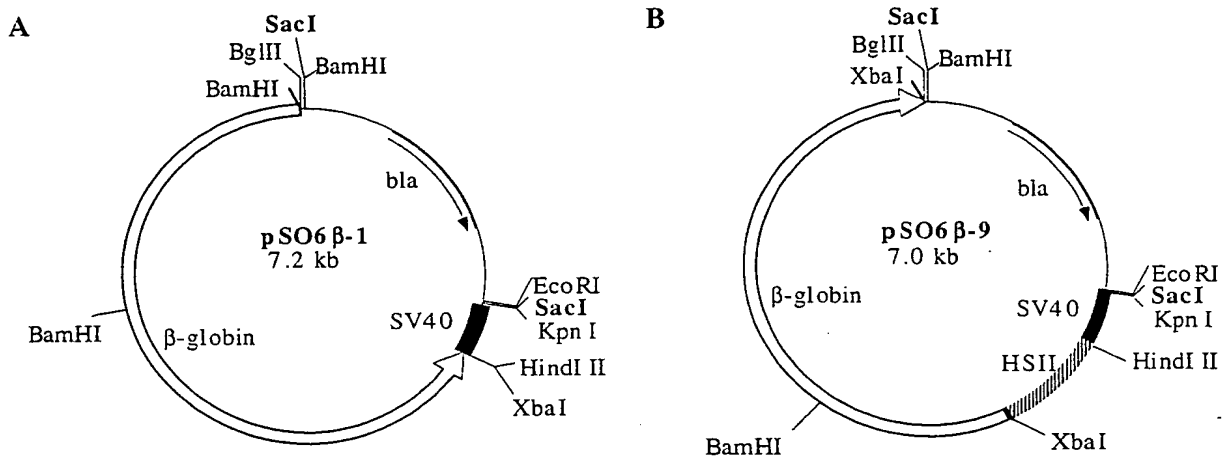

FIG. 1. Structure of β-globin plasmids. (A) pSO6β-1 contains the *bla* gene (ampicillin resistance), derived from pBR322 (coordinates 2369–4362). The SV40 fragment is from the HindIII site at coordinate 5172, through the *ori* region, to the KpnI site at coordinate 294 (366 bp). The β-globin fragment is from the BglII site at coordinate 60577 to the XbaI site at coordinate 65421 in a reverse orientation relative to the SV40 early promoter. The fragments were joined using synthetic linkers carrying the restriction sites shown in the map. The detailed procedure of the construction has been described previously (25). (B) Plasmid pSO6β-9 (26) has the same pBR322 and SV40 sequences as pSO6β-1. The β-globin gene fragment is from HpaI (coordinate 61322) to BglII (coordinate 65558), cloned at the same orientation as the SV40 early promoter. The HSII fragment is from the HindIII at coordinate 8487 to XbaI at coordinate 8860. The fragments were joined using synthetic linkers carrying the restriction sites shown in the map.

hemoglobin-containing cells were scored as previously described (29).

Isolation and analysis of β-globin mRNA

Total RNA was harvested from 4–10 × 10^5 cells by acid guanidinium thiocyanate-phenol-chloroform extraction (30). RT was performed with 200 ng total RNA, using a Reverse Transcriptase System kit (Promega, Madison, WI). One fifth of each RT product was amplified by PCR in a total volume of 50 μl for 35 cycles (92°C for 1.5 min, 60°C for 1 min, and 72°C for 1 min). Aliquots of the reaction products (one-fifth volume) were analyzed by gel electrophoresis and Southern blotting with [^{32}P]-labeled allele-specific oligonucleotide probes. The following primers were used: P-4: 5′-CACCGAGCACTTTCTTGCCA-3′ from nucleotides 62527–62508; FS-8: 5′-CTCCTGAGGAGAAGTCTGC-3′ from nucleotides 62200–62218. β-globin coordinates are from GenBank accession number j00093.gb_pr. Allele-specific oligonucleotide hybridization and GATA-1 mRNA analyses were performed as previously described (31,32).

RESULTS

A series of β-globin plasmids carrying various regulatory elements were prepared. The initial constructs, represented here by pSO6β-1 (Fig. 1A), carried the complete β-globin gene and ~1.5–2.0 kb flanking sequences. Experiments with those plasmids showed efficient gene transfer into hematopoietic cells and a low level of β-globin expression (not shown). We then prepared con-

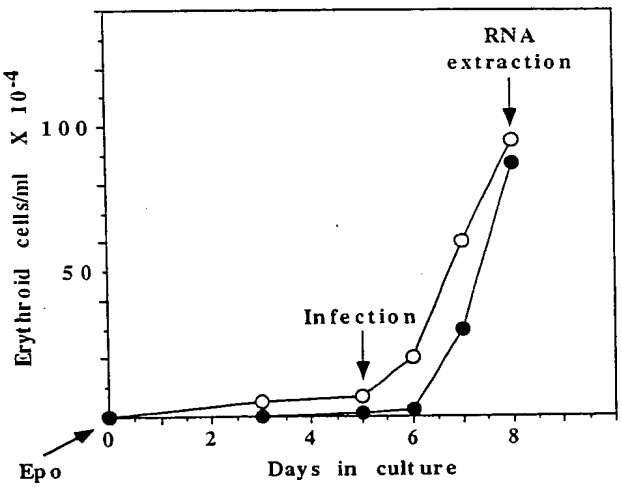

FIG. 2. Growth and differentiation of cultured erythroid cells derived from a patient with β^0-thalassemia. EPO was added on day 0. The cells were infected with β-globin pseudovirions on day 5, and total RNA was harvested on day 8. Open circles indicate the number of total erythroid cells, and closed circles indicate the number of hemoglobinized cells. Cells of β^0-thalassemia patients are devoid of HbA, but contain HbF and HbA_2.

structs, including an 882-bp DNA fragment carrying the human LCR HSII element upstream to the β-globin gene, which was shown to enhance β-globin expression in transgenic mice (33). Constructs with and without the β-globin 3′ enhancer and with and without the SV40 enhancer were tested. We found that the presence of the entire HSII element greatly interfered with packaging as SV40 pseudovirions. Therefore, these constructs were not further studied. pSO6β-9 (Fig. 1B), which carries only the 5′-end of HSII, allowed packaging, although at a low efficiency (titers obtained were $\sim 10^4$ IU/ml).

Expression studies were performed using erythroid cells derived from the peripheral blood of β^0-thalassemia patients. Soon after the addition of EPO to the cultures, the erythroid cells start to proliferate (Fig. 2, open circles) and differentiate (hemoglobinized cells, closed circles). The cells were infected on day 5 and harvested on day 8, when globin mRNA level reaches its peak (23).

A limiting factor in these experiments is the number of cells available for infection. Therefore, the number of variables tested in each experiment was small. We first asked if the 5′ HSII element enhances β-globin gene expression. β-globin mRNA was detected by RT-PCR, and the products were hybridized to allele-specific oligonucleotide probes to distinguish between endogenous (β^0-thalassemia) and exogenous (normal) messages. In the experiment shown in Figure 3A, the RNA of cultures derived from both patients studied, R.D. and M.D., yielded a signal of similar intensity with the mutant probe, indicating expression of the endogenous β-globin gene. The culture of M.D. infected with SO6β-9 pseudovirions showed a significant level of signal with the wild-type (wt) probe. Cells of R.D. were infected with both SO6β-9 and SO6β-1. The wt signal obtained with SO6β-9 was 2–3-fold higher than that obtained with SO6β-1, although the MOI with SO6β-9 was 10-fold lower. Therefore, the presence of the LCR fragment in SO6β-9 increased the expression of β-globin 20–30 times. As expected, RNA harvested from a control normal culture, derived from a nonthalassemic individual, gave a signal only with the wt probe. The intensity of the signal was several-fold higher than that of the signal seen with the thalassemic cells infected with SO6β-9. The MOI with SO6β-9 was 0.1; that is, at most, only 10% of the cells were infected, suggesting that increased production of total β-globin mRNA in the culture by at least several-fold may be achieved by increasing the MOI.

Experiments with cultures obtained from 2 additional thalassemia patients also demonstrated that infection with SO6β-9 pseudovirions consistently led to elevated expression of the normal β-globin signal, in comparison to mock-infected and SV40-infected cells (not shown).

In additional experiments, we have used as an internal standard for erythroid-specific RNA levels RT-PCR assays of the erythroid-specific gene GATA-1 (Fig. 3C). Expression of the exogenous, normal β-globin gene was significant in cultures derived from both patients, S.K. and Z.K. Importantly, the level of expression depends on the MOI (Fig. 3D). Figure 3D also suggests that infections using larger volumes are less efficient.

Cultures derived from both patients, S.K. and Z.K., show a low level signal for β-globin expression in the control experiments (SV40-infected and mock-infected, respectively). This is most likely due to the presence of δ-globin or γ-globin mRNA or both. The δ-globin and γ-globin genes have high similarity to the β-globin gene, and the primers for the RT-PCR and the oligonucleotide probe used in the hybridization recognize the δ-globin and the γ-globin gene at almost the same efficiency as the β-globin gene, causing a cross-reaction. Control experiments with noninfected cells of several β^0-thalassemia patients, which were cultured separately, also showed a low-level signal with the wt probe, indicating that this is not due to contamination by β-globin pseudovirions.

DISCUSSION

The present study demonstrates the potential feasibility of developing SV40 pseudovirions for ex vivo gene therapy of β-thalassemia. In the transient expression experiments reported here, the inserted gene was expressed at a significant level in primary human erhythroid cells. The expression was directly related to the MOI, suggesting that infection at an MOI of 1–5 IU/cell, when almost every cell in the culture is infected, will yield a transient expression level similar to that of the normal control. Importantly, control assays of the endogenous β-globin (mutant) RNA and GATA-1 mRNA showed that the cells were not adversely affected. These results and those of previous studies (22) suggest that SV40 merits additional studies as a vector for diseases affecting the BM (34).

In agreement with previous studies, the results presented here demonstrate that the 5′ HSII element enhances expression of the linked β-globin gene 20–30-fold in differentiating human erythroid progenitors (35).

Long-term expression of genes delivered via SV40 pseudovirions has not been rigorously tested. However, recent studies have shown stable expression for many weeks for the duration of the experiments: 6 weeks in NIH3T3 cells (22), 17 weeks in various mouse tissues (36), and over 20 weeks in human 293 and HT1080 cells (N. Shimron and A. Oppenheim, unpublished observations, 1999). It is possible that in order to achieve efficient integration of the inserted gene, additional elements will be required. This subject is currently under investigation in our laboratory.

Our recently developed method for in vitro packaging

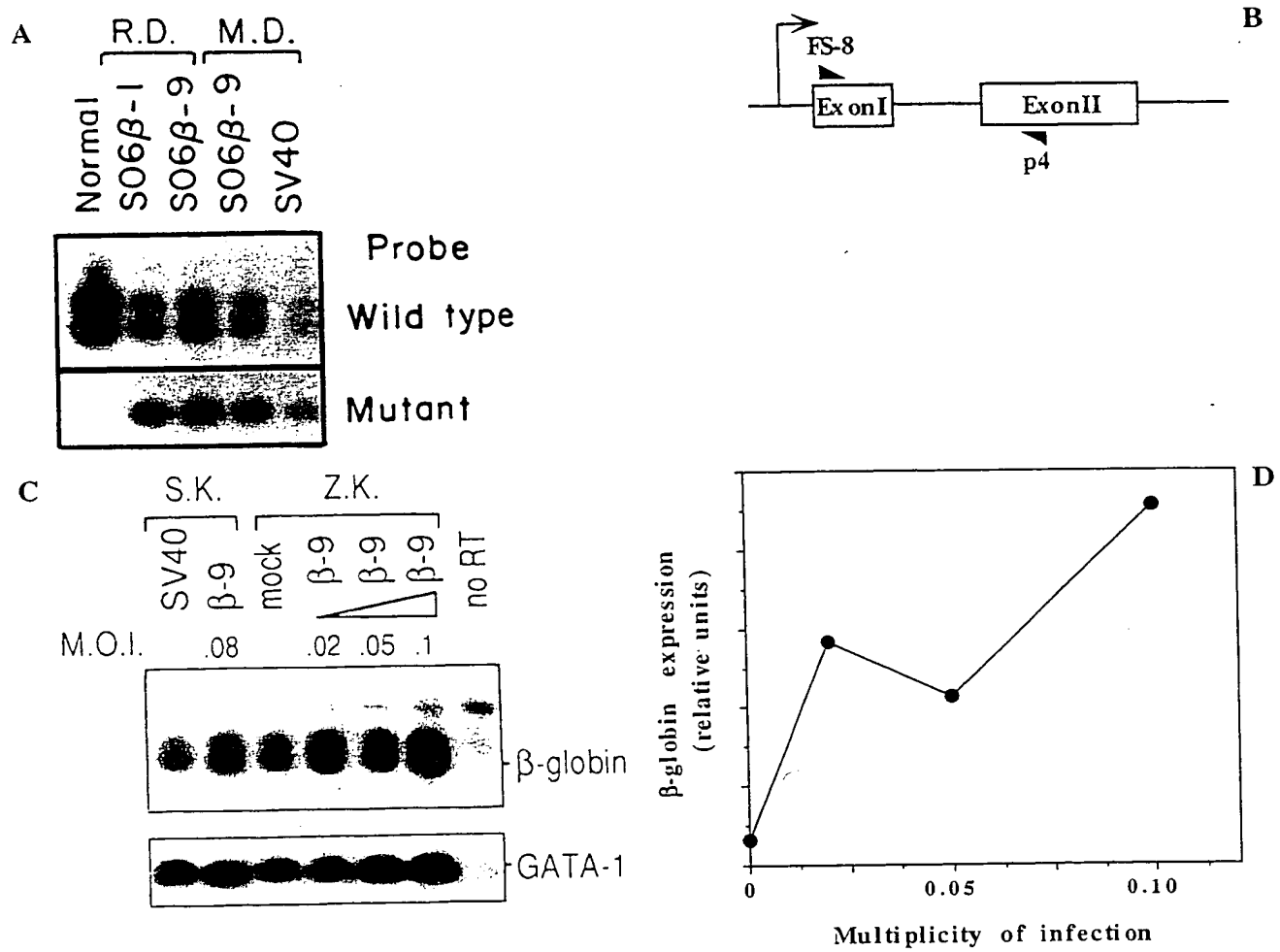

FIG. 3. (A) Expression of the exogenous normal β-globin gene in erythroid cells derived from β^0-thalassemia patients. Total RNA harvested from erythroid cultures of 2 patients (as designated at top) was assayed for β-globin expression. Both patients are homozygous for a frameshift mutation in codon 44 (–C). The PCR products were analyzed by Southern blotting with β-globin wt or mutant probe, as indicated at right. Infection with SO6β-1 was performed at an MOI of 1 and with SO6β-9 at an MOI of 0.1. RNA harvested on day 8 from an erythroid culture of a normal individual was analyzed as a control (Normal). (B) Position of the primers used for the RT-PCR analyses. The diagram shows a portion of the β-globin gene and the position of the primers. (C) Total RNA harvested from erythroid cultures was assayed for β-globin and GATA-1 mRNA as previously described (32). Cultures of progenitors derived from Z.K. were infected at different MOI. as indicated at the top. GATA-1 analysis served as an internal standard for the RNA level. Mock was infected with a freeze-thaw extract of a mock-transfected COS culture, treated in parallel to the pseudoviral stock. (D) Effect of MOI on β-globin expression. The results for Z.K. shown in C were quantified by scanning the autoradiogram. Infection at an MOI of 0.02 was performed with 0.4 ml pseudoviral stock per 2×10^5 cells, at an MOI of 0.05 with 1 ml per 2×10^5 cells, and at an MOI of 0.10 with 0.4 ml per 2×10^5 cells using a pseudoviral stock that had been concentrated 5-fold. As seen, infection at a higher volume is less efficient.

of helper-free pseudovirions (37) combines the efficiency and gene delivery characteristic of viral vectors with the safety and flexibility of nonviral delivery systems. Viral packaging in vitro allowed packaging of significantly larger plasmids (at least 7.5 kb) with minimal SV40 sequences (150 bp). The upper limit of plasmid size for packaging in vivo is ~5.4–5.7 kb (26,38). In addition, in vitro packaging allows the inclusion of the β-globin LCR elements. The LCR HSII 0.4 kb element of pSO6β, which reduced packaging in the present work by 20-fold, did not affect packaging in vitro (37). Furthermore, a larger, 0.9-kb HSII element had no adverse effects on plasmid packaging in vitro. In vivo, this HSII element reduced packaging to below detection level (26). This suggests

that additional LCR elements, anticipated to allow higher-level and appropriately regulated β-globin expression, may be included. Most importantly, in vitro, the presence of a helper virus is no longer required, and packaging can be performed under utmost controlled conditions, which will allow for the use of SV40 psuedovirions in human clinical trials.

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Inhibition of HIV-1 by an anti-integrase single-chain variable fragment (SFv): delivery by SV40 provides durable protection against HIV-1 and does not require selection

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Human immunodeficiency virus type 1 (HIV-1) encodes several proteins that are packaged into virus particles. Integrase (IN) is an essential retroviral enzyme, which has been a target for developing agents to inhibit virus replication. In previous studies, we showed that intracellular expression of single-chain variable antibody fragments (SFvs) that bind IN, delivered via retroviral expression vectors, provided resistance to productive HIV-1 infection in T-lymphocytic cells. In the current studies, we evaluated simian-virus 40 (SV40) as a delivery vehicle for anti-IN therapy of HIV-1 infection. Prior work suggested that delivery using SV40 might provide a high enough level of transduction that selection of transduced cells might be unnecessary. In these studies, an SV40 expression vector was developed to deliver SFv-IN (SV(Aw)). Expression of the SFv-IN was confirmed by Western blotting and immunofluorescence staining, which showed that >90% of SupT1 T-lymphocytic cells treated with SV(Aw) expressed the SFv-IN protein without selection. When challenged, HIV-1 replication, as measured by HIV-1 p24 antigen expression and syncytium formation, was potently inhibited in cells expressing SV40-delivered SFv-IN. Levels of inhibition of HIV-1 infection achieved using this approach were comparable to those achieved using murine leukemia virus (MLV) as a transduction vector, the major difference being that transduction using SV40 did not require selection in culture whereas transduction with MLV did require selection. Therefore, the SV40 vector as gene delivery system represents a novel therapeutic strategy for gene therapy to target HIV-1 protains and interfere with HIV-1 replication.

Keywords: *HIV-1; SV-40; gene therapy; intracellular immunization*

Introduction

Various techniques have been developed to express recombinant constructs within cells, in culture, in animal models and in humans.[1] The application of molecular genetics to human biology and disease has improved our understanding of and ability to treat a variety of disease states. Retroviral gene delivery vectors based on oncoretroviruses, such as Moloney murine leukemia virus (MLV), have been the most commonly used vectors for gene transfer into the host cell genome.[2,3] MLV has been used to deliver therapies for diverse diseases, including cancer, inherited genetic disorders and infection with HIV-1. However, onco-retroviral vectors have limitations that often include a need to select cultured cells to enrich for those cells expressing the transgene. Such selection generally necessitates transduction *ex vivo*, followed by cumbersome selection in culture.

We have devised a gene transfer system based on simian virus-40 (SV40) as a vector.[4] SV40 infects a wide range of cell types from humans and other mammals and expresses its genes in them. Recombinant, replication-deficient SV40-derived vectors may express transgenes stably in cell lines, in primary cultures, and *in vivo*.[4–6] The virus is stable to manipulation and can be made to high titer (≥10^{10} infectious units (IU)/ml), and further concentrated if needed.

HIV-1, as a member of the lentivirus family, has a complex viral life cycle and utilizes multiple cellular and virally encoded regulatory proteins to tightly control its replication.[7] The essential retroviral enzymes, reverse transcriptase (RT), ribonuclease H (RNaseH), protease (PR) and integrase (IN), lack cellular counterparts and have been used as targets for developing agents that inhibit virus replication.[8–10] Despite considerable advances in anti-RT and PR chemotherapy, genetic changes in the virus can confer drug resistance.[11] This problem has led to proposals for alternative therapeutic strategies in which host cells may be genetically altered or engineered to confer long-lasting protection against virus infection or replication.[12,13] Several such strategies are currently being reported and applied to the inhibition of HIV-1 replication. They include exploitation of *trans*-dominant-negative mutant HIV-1 protein expression, viral antisense oligonucleotide sequences, specific

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ribozymes, HIV-1 *trans*activated suicide genes and intracellular antibodies against several different HIV-1-specific proteins.[14–26]

Recently, we reported that intracellular expression of SFv moieties targeted to RT, IN and Rev strongly inhibited HIV-1 replication in human cells.[15–20,23,24,27] An early event in the life cycle of all retroviruses, including HIV-1, is integration of a double-stranded proviral DNA into the host cell genome. This step is necessary for productive viral replication.[28] In natural infection, linear viral DNA contained within pre-integration complexes is the direct precursor of the integrated proviral genome.[29] In previous studies, we demonstrated that anti-IN SFv #33, driven by the cytomegalovirus immediate–early promoter (CMV-IEP) and delivered by MLV to selected human T-lymphocytes, effectively inhibited HIV-1 replication.[20] To assess intracellular immunization as a tool for gene therapy of HIV-1 infection further, IN was targeted for specific blockade by the same anti-IN intracellular SFv expression construct, but delivered using an SV40 vector. The effectiveness of this construct in inhibiting HIV-1 was tested in a CD4$^+$ human T lymphoma cell line.

We show here that an SV40-derived vector can transduce human T-lymphocytic cells to express the protein SFv-IN effectively without selection. Furthermore, HIV-1 replication, as measured by HIV-1 p24 antigen expression and syncytia formation, was inhibited in SupT1 T-lymphocytic cells expressing SFv-IN.

Results

Construction of viruses expressing SFvIN#33 and HBsAg proteins

A map of SV(HBS) has been reported.[30] This recombinant SV40 virus was used as a negative control. Construction of pSLXCMV-SFvIN#33, which expresses SFvIN#33, has been described previously.[20] Briefly, sequences encoding the variable light (V_l) and variable heavy (V_h) chains of the anti-IN monoclonal antibody (Mab) were cloned from a murine hybridoma cell-line's RNA. After ligation of V_l and V_h chains into a single fragment, by utilizing the flexible linker $(GGGGS)_3$, the SFv fragment was cloned into an SV40 expression vector downstream of cytomegalovirus immediate–early promoter (CMV-IEP). The orientations and structures of open reading frames were verified by DNA sequencing. A map of the SV40 vector containing the SFvIN#33 (SV(Aw)) driven by CMV-IEP, and used for all these studies, is shown in Figure 1.

Detection of SFvIN#33 by Western blotting

Lysates were prepared from 10^6 SupT1 cells transduced as described with SV(Aw) or with MLV-SFvIN#33, or mock-transduced. These proteins were electrophoresed using SDS-PAGE, blotted to PVDF membranes, probed with anti-mouse IgG antibody and visualized as described in Materials and methods. As a positive control, recombinant SFvIN#33 produced in *E. coli* was used. Results demonstrated that both selected MLV-SFvIN#33-transduced SupT1 cells and unselected SV(Aw)-transduced cells expressed the SFvIN#33 transgene (Figure 2). Levels of transgene expression were comparable in unselected SV(Aw)-transduced cells and in selected MLV-SFvIN#33-transduced cells (Figure 2). The larger molecular size bands seen here in the extracts from

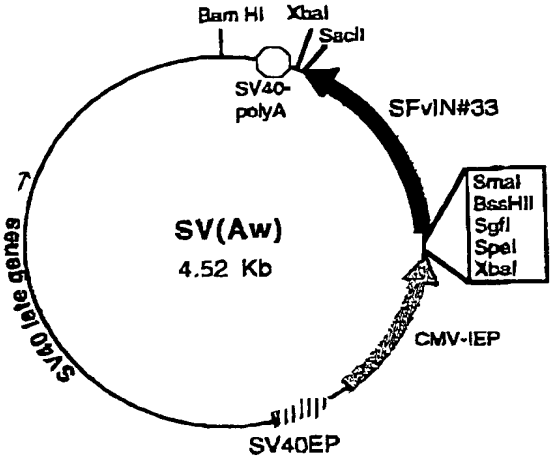

Figure 1 Schematic map of the SV40-derivative construct used to express SFvIN#33. The genome of SV(Aw), the SV40-derivative used to transduce the SFvIN#33, is illustrated here. This virus was constructed as described in Materials and methods, and in general according to the approach outlined in previous publications.[6] Briefly, in this virus, the SFvIN#33 cDNA is driven by the CMV-IEP, which is in turn immediately downstream from the SV40-EP. The latter overlaps the SV40 origin of replication, and thus cannot be entirely deleted from the viral genome. The late virus genes (VP1, VP2, VP3) are intact in this construct, as are the SV40 late promoter, enhancer and early and late polyadenylation signals. The construction of SV(HBS), the control SV40 virus used in these studies, was reported previously.[30]

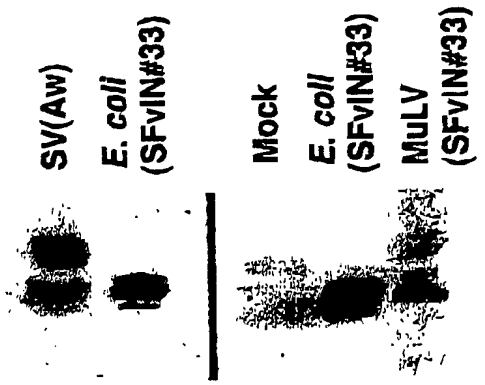

Figure 2 Expression of anti-IN SFv in SV(Aw)- and MLV-SFvIN#33-transduced cells. SupT1 cells were treated with SV(Aw), once at MOI of 10, without selection. Protein from 10^6 cells, harvested 4 days after transduction, was electrophoresed on SDS-PAGE, blotted to PVDF membranes and probed with anti-mouse IgG to visualize expression of the SFvIN#33. In parallel, protein from an equal number of selected, MLV-SFvIN#33-transduced cells, was electrophoresed, blotted and visualized similarly. For both preparations, SFvIN#33 expressed in E. coli was the positive control. Equal numbers of non-transduced SupT1 cells were the negative control.

SV(Aw)-transduced cells may represent either splice variants of the original transcript or ribosomal reading through translational stop signals.

SV40 delivery of SFvIN#33 and expression in mammalian cells

To obtain transfectants expressing SFvIN#33 and HBsAg, recombinant SV40-based viruses were prepared from COS-7 packaging cells and SupT1 cells were treated with these viruses as described in Materials and methods. After infection with SV(Aw) or SV(HBS), the cells were cultured for 2 weeks without selection and then analyzed by immunostaining. Expression of SFvIN#33 was detected using an anti-mouse IgG. The SV(Aw)-treated SupT1 cells express the transgene, SFvIN#33 (Figure 3). SupT1 cells treated with SV(HBS) were used as controls and did not express detectable SFvIN#33 (Figure 3). The anti-IN-SFv protein was principally detected in the cytoplasm of transduced cells.

These results are important for several reasons. First, these data demonstrate that SV40 can transduce SupT1 cells to express the anti-IN SFv efficiently. Secondly, the efficiency of SV40 transduction is very high: more than 90% of cells stain positively for SFvIN#33 expression. Unlike our experience with MLV transduction of the same SFv (approximately 30% of cells were positive for SFvIN#33 expression), selection of the SV40-treated SupT1 cells for transgene expression in culture was not required to achieve very high rates of transduction. Finally, SV40 transduction did not slow the rate of cell proliferation significantly. Cell recovery, tested by counting of trypan blue-negative cells, was comparable in SV40-treated and MLV-treated cultures (data not shown). Thus, SV40 transduction was not demonstrably toxic to these cells.

Inhibition of HIV-1 replication in human T-lymphoid cells that express the anti-IN SFv: comparative effects of MLV and SV40 transduction

We then tested whether intracellular expression of SFvIN#33 delivered by SV40 to SupT1 cells could prevent HIV-1 replication in this line of susceptible T-lymphoid cells, compared to the same SFv delivered by MLV-SFVIN#33. HIV-1 challenge studies were performed using virus strain NL_{4-3}. Control SupT1 cells were untreated, or treated with SV(HBS). It is of note that cell growth curves and viability of SupT1 cells were unaltered by transduction with either of these SV40-derived vectors, as described above. The infectivity assays were performed with transduced, mixed SupT1 cell populations. For comparison, MLV-SFvIN#33-transduced, selected SupT1 cells were challenged in parallel.

For HIV-1 challenge experiments, unselected and uncloned SFvIN#33-transduced SupT1 cells, HBSAg SupT1 cells and non-transduced SupT1 cells were treated with two different doses of HIV-1_{NL4-3} (0.05 pg/ml and 0.5 pg/ml of p24 antigen equivalents). Replication of HIV-1 in these cultures was evaluated by quantifying the levels of HIV-1 p24 antigen released into the culture medium (Figure 4). At the lower MOI, only very low levels of HIV-1 p24 antigen were observed in the supernatants of SV(Aw)-transduced, unselected SupT1 cells challenged with HIV-1_{NL4-3}. Similar protection was seen in selected SupT1 cells transduced with MLV-SFvIN#33, that had been selected following transduction to assure sustained transgene expression. These data indicate virtually complete protection from HIV-1 by SV40 transduction of SFvIN#33, at levels comparable to those achieved using the same anti-IN-SFv delivered with MLV-SFVIN#33. This protection was evident throughout the time-course of our studies, up to and including 24 days after challenge.

Control non-transduced and SV(HBS)-transduced cells showed no inhibition of HIV-1 replication, as demonstrated by dramatic initial increases in HIV-1 p24 antigen (Figure 4). On day 24 after infection, SupT1 SFvIN#33 mixed cell populations showed complete inhibition of HIV-1 p24 antigen production, as compared with the non-transduced SupT1 cells and cells expressing HBsAg. At the higher MOI, little or no protection from HIV-1 challenge was seen in cultures expressing SFvIN#33 transduced by either MLV-SFvIN#33 or SV40 (data not shown).

Microscopically, SV(Aw)-transduced SupT1 cells

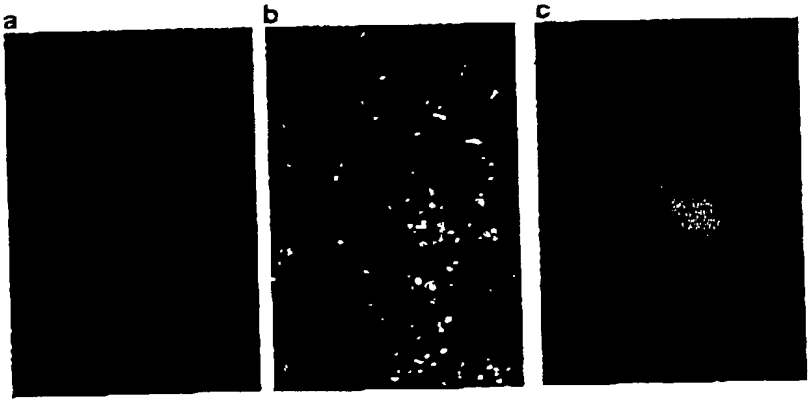

Figure 3 Analysis of SFv protein expression in transduced SupT1 cells. Transduced SupT1 cells were fixed and immunostained with goat anti-mouse polyclonal IgG, using a FITC-conjugated rabbit anti-goat IgG. SupT1 cells transduced with SV(HBS) (a) were used as negative controls. SupT1 cells transduced with SV(Aw) (b, c) demonstrated mainly cytoplasmic staining. Magnification X100 (a, b) and x400 (c).

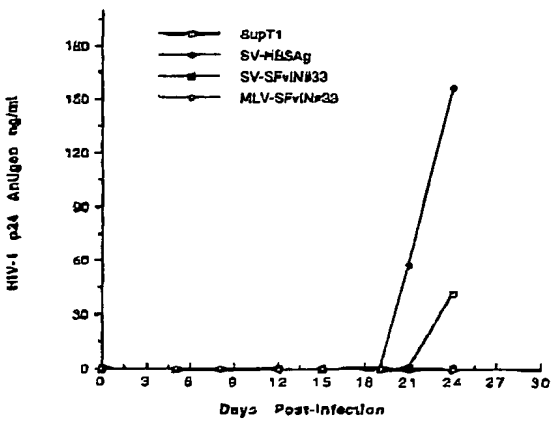

Figure 4 Inhibition of HIV-1 replication in SFvIN-transduced T-lymphoid cells. SupT1 cells were transduced with SV(Aw), or SV(HBS) (mixed cellular populations). These SupT1 cells were challenged with HIV-1_{NL4-3} virions with identical quantities of HIV-1 p24 antigen, overnight. HIV-1 replication was quantified by assaying HIV-1 p24 antigen levels in the culture supernatants by ELISA. These graphs are representative of two independent experiments.

showed weak cytopathic effects when infected with HIV-1_{NL4-3} as assessed by observing syncytium formation and cell death (Figure 5). Even when cultures were challenged at high HIV-1_{NL4-3} MOI, cells transduced with SV (Aw) showed delayed syncytium formation at assay points up to 18 days after challenge (Figure 5). These results suggest that intracellular anti-IN SFv expression protected cells against the cytopathic effects of HIV-1. On day 21 after infection, non-transduced SupT1 cells or SV(HBS)-transduced SupT1 cells, infected with HIV-1_{NL4-3} started to die. By comparison, SupT1 cells transduced with SFvIN#33 showed normal cell growth. Eventually, however, challenge at 0.5 pg/ml doses of HIV-1_{NL4-3} overcame the protective activity of SFvIN#33, whether delivered by SV40 or by MLV (not shown).

Discussion

We have previously reported that anti-IN SFv#33 significantly inhibited HIV-1 replication, when delivered to SupT1 cells using a retroviral vector, followed by selection for SFv-expressing cells. We now report that anti-IN SFv#33 delivery, using SV40 as a transduction vehicle, is equally effective as MLV SFvIN#33 in protecting SupT1 cells from HIV-1 infection and that this delivery system has the distinct advantage that its effectiveness does not require selection for transgene-expressing cells before challenge with HIV-1.

SV40 was chosen as a delivery vehicle to compare with MLV in these studies for several reasons. SV40 infects virtually all mammalian nucleated cell types, and high titer SV40-derived gene transfer vectors are stable and are easily produced, with expression of SV40-delivered transgenes persisting for long periods *in vivo*.[4,5] Also, a single transduction treatment of normal human or simian bone marrow progenitor cells results in long-term carriage of the transgene, most likely via integration (DS Strayer *et al*, preliminary observations). SV40 also transduces resting and dividing cells with about equal efficiency: unstimulated peripheral blood mononuclear cells are easily transduced *ex vivo*, as are hepatocytes, neurons and other non-cycling cells *in vivo*,[5,6] (M Zern and D Strayer, in preparation; R Chowdhury and D Strayer, in preparation). This is of importance, as non-activated T-lymphocytes and non-proliferating monocyte/macrophages are critical cellular reservoirs for HIV-1 *in vivo*.

The SV40-derived constructs applied to these studies are replication-incompetent in cells that lack SV40 Tag.[4] In addition, the viral large T antigen is the major target of the immune system in eliminating cells infected with wild-type (wt) SV40,[31–35] suggesting that Tag-deleted, replication-defective SV40-derivative viruses should be minimally immunogenic. We have confirmed this hypothesis: such vectors can be administered to normal, immunocompetent animals many times but do not elicit detectable neutralizing antibody *versus* SV40.[30]

The combination of lack of immunogenicity and high levels of durable transduction efficiency of SV40 for bone marrow-derived cells, suggested that this virus might

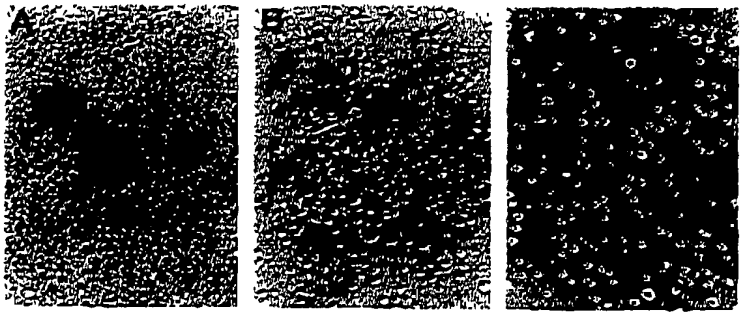

Figure 5 Anti-IN SFv inhibition of cytopathic effects of HIV-1 infection. The microscopic morphology (syncytium formation and cell death) of SupT1 cells infected with syncytium-inducing strain HIV-1_{NL4-3} (0.5 pg/ml) after 15 days after infection is shown. (a) SupT1 non-transduced cells; (b) mixed cell populations expressing HBsAg; (c) mixed cell populations transduced with SV(Aw) and expressing anti-IN SFv. All photomicrographs are ×400.

deliver anti-HIV-1 gene therapeutics effectively. To test this possibility, we developed a recombinant SV40 vector to deliver the SFvIN#33, which had already been shown to inhibit HIV-1 infection when it was delivered by MLV.[20]

Cytoplasmic SFvIN#33 blocks HIV-1 infection somewhat more efficiently than a nucleus-targeted SFvIN#33.[20] Our current immunostaining results suggest that the SFvIN#33 localizes predominantly in the cytoplasm. Using SV40 to express the anti-IN SFv#33 did not affect the localization of the protein, which is still cytoplasmic. In this report, we demonstrate that intracellular expression of an anti-HIV-1 IN-SFv decreases HIV-1 replication in T-lymphocytic cells. This finding is significant in that it suggests a new and previously unexplored avenue in attempts to inhibit HIV-1 replication before integration of viral DNA into the host genome.

Transduction using onco-retroviral vectors has certain limitations. Firstly, one must often select the cells expressing the gene of interest by using a neomycin-resistance gene (*neo*^{r}). Secondly, although they may vary in host cell range depending on how they are packaged, oncoretroviruses usually infect a limited range of cells and these cells must be dividing at the time of infection. Finally, the efficiency of transduction with MLV vectors in our hands is relatively low. In contrast, SV40 infects a wide range of cells and has a very high transduction efficiency. Our results show that >90% of cells treated with SV(Aw) express anti-IN SFv#33 after transduction and continued culture. This is an advantage that could be very useful for transduction of primary cells. It has been reported that SV40 can infect such cells as hematopoietic and peripheral blood mononuclear cells (PBMC).[5,36] The authors in the former report used the SV40-based pseudoviral system for transfer of the human MDR1 gene. Highly efficient gene transfer into several important cell types, including fresh unmanipulated primary human bone marrow cells and PBMC, was shown.[5,36] We have also found high efficiency gene transfer to bone marrow-derived cells using SV40, as described above (DS Strayer *et al*, in preparation).

Of importance, most if not all anti-HIV-1 gene therapeutics can be overwhelmed by utilizing relatively high challenge MOIs dramatically *in vitro*. This observation was repeated in our studies: SFvIN#33 delivered by both SV40 and MLV was highly effective in inhibiting HIV-1 at challenge 0.05 pg/ml of p24 antigen, but did not dramatically alter the course of HIV-1 infection at challenge 0.5 pg/ml of p24 antigen. This finding may be problematic for inhibiting HIV-1 in the interstices of lymphatic tissues of infected individuals, where HIV-1 concentrations may be higher than those inhibited by the therapeutic approaches described here.[37] In such cases, multiple simultaneous therapeutic modalities or multiple administrations may be necessary to provide effective protection.

The ability to deliver functional anti-HIV-1 SFv-IN, using SV40 as vector represents a novel and potentially useful technology that could contribute to the efficient inhibition of HIV-1 replication. The high efficiency of SV40 transduction, the lack of need for selection, the ability of SV40 to transduce a wide range of target cells and the potential for multiple administrations *in vivo*, all combine with the effectiveness of SFvIN#33 delivery by SV40 demonstrated here, to suggest that SV40-delivered anti-retroviral gene therapy represents a potentially useful new class of anti-retroviral agents. This concept could also further assist in analyzing the molecular events within the lentiviral life cycle.

Materials and methods

Plasmids and viral expression constructs

The HIV-1 molecular clone used in this study was pNL_{4-3}. This strain was obtained from the AIDS Reagent Repository (NIH). $HIV\text{-}1_{NL4-3}$ is T-tropic viral strain with highly cytopathic effects.

The construct, MLV vector containing anti-IN #33 (pSLXCMV-SFvIN#33), has been described previously.[20] The IN#33 SFv binds to the non-specific DNA binding site in HIV-1 IN, and strongly inhibits HIV-1 replication in transduced human T-lymphocytic cells.

Construction of recombinant SV40 derivative viruses for gene transfer has been described previously in principle.[4] The recombinant SV40-derived virus, SV(Aw), was made similarly and will be described briefly. The wild-type (wt) SV40 genome cloned into pBR322 was provided as plasmid pBSV-1 from Janet Butel (Baylor College of Medicine). The large T antigen gene was excised from this plasmid and replaced by a polylinker. Into this polylinker, an expression construct containing cytomegalovirus intermediate-early promoter (CMV-IEP) plus the cDNA for SFvIN#33 was cloned, to yield pBSV(CMV)Aw.

The control virus used for these studies was SV(HBS). The production and characterization of this virus have been reported.[30] Briefly, the gene for hepatitis B surface antigen (HBsAg) was cloned into the Tag-deleted SV40 genome, immediately downstream from two tandem SV40 early promoters. The resulting plasmid was pSV5(HBS).

Production of SV40 derivative viruses

The techniques used in generating viruses from such plasmids as pSV5(HBS) and pBSV(CMV)Aw have been described.[4] Briefly, the virus genome was excised from the carrier plasmid and purified by agarose gel electrophoresis. It was then recircularized and transfected into COS-7 cells, the packaging cell-line used for these studies. No helper virus was used: the COS cells supply the necessary Tag *in trans*. These virus stocks were prepared as cell lysates. After initial virus preparations were made in this fashion, expanded stocks were prepared by infecting COS-7 cells with virus from the original preparation. Further transfections are not done. Resulting viruses are replication incompetent.[4]

Virus stocks are titered by *in situ* PCR as described previously.[38] This provides for direct enumeration of infectious units in a preparation.

Cell cultures

The COS-7 packaging cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal calf serum (FCS, Hyclone, Logan, UT, USA). The same culture conditions were used for 293T. SupT1, a CD4+ human T-lymphocyte cell line susceptible to HIV-1 infection, was grown in RPMI-1640 medium + 10% FCS.[4] All the cells were grown at 37°C in humidified incubator with 5% CO_2.

HIV-1 viral stocks

293T cells were transfected by a standard calcium-phosphate method (Promega, Madison, WI, USA). 1×10^6 293T cells were plated in 10 cm dishes, transfected with 10 mg of pNL_{4-3} DNA and then incubated in DMEM containing 10% FCS (growth medium) for 7 h. The medium was then removed and replaced with fresh DMEM growth medium + 10% FCS. Virus-containing supernatants were collected at 48 h. The quantity of virus present in transfected cell supernatants was determined by measuring HIV-1 p24 antigen levels, using an enzyme-linked immunosorbant assay (ELISA) (Cellular Products). Viral stocks were assayed for their infectious titers on CEM T-cells, and these data were used to calculate MOI in each experiment.

Transduction of T-lymphocytic cells

Procedures for infecting SupT1 cells with MuLV-SFvIN#33, and for selecting transgene-expressing transduced cells, have been reported elsewhere in detail.[20] For transduction using SV40-derived vectors, SupT1 cells were treated with SV(Aw), carrying the cDNA for SFvIN#33, or SV(HBS), carrying the gene for hepatitis B surface antigen (HBsAg) for 24 h at an MOI of 10. This step was repeated twice, but at MOI of 3, on sequential days. No selection was used. The SV40-treated SupT1 cells were then cultured for 2 weeks.

HIV-1 challenge

The mixed populations of HIV-1-susceptible T-cells (SupT1) which expressed the SFvIN#33 and HBSAg proteins were maintained in RPMI-1640 + 10% FCS for at least 2 weeks before HIV-1 infection. Parental SupT1 cells alone and cells expressing the SFvIN#33 and HBSAg proteins, were incubated with infectious cell-free $HIV\text{-}1_{NL4\text{-}3}$ overnight. The cells were washed with prewarmed, serum-free medium and then maintained in growth medium. Every 3 days, cells were split 1:2 to maintain a cell density of approximately 10^6/ml and the culture supernatants were collected for HIV-1 p24 antigen analyses. The HIV-1 p24 antigen levels in supernatants were determined by ELISA. Cell viability was monitored by trypan blue exclusion.

Immunostaining for protein expression

Subcellular localization of intracellularly expressed SFvIN#33 protein within the cells was determined by indirect immunofluorescence assays. Infected SupT1 cells were cultured after infection with SV40 on eight-chambered glass slides overnight. Cells were then fixed with methanol at room temperature for 10 min and blocked overnight with 1% phosphate-buffered saline/bovine serum albumin (PBS/BSA). The slides were treated overnight with a 1:200 dilution of goat anti-mouse polyclonal IgG (Nordic Immunology, Tilburg, The Netherlands). After several washings, cells were incubated with a 1:64 dilution of fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat IgG (Sigma, St Louis, MO, USA) for 1 h at 37°C. After washing five times in PBS, cells were mounted and analyzed by epifluorescence microscopy.

Detection of SFvIN#33 protein by Western blot analysis

Cells (10^6), which had been transduced with MLV or SV40 vectors carrying the SFvIN#33 expression construct, were lysed with lysis buffer (100 mM Tris pH 8.0, 100 mM NaCl, 0.5% NP-40, 1 mM PMSF, 10 mg/ml leupeptin, 2 mg/ml aprotinin, 10 mg/ml pepstatin). Protein lysate was loaded on a 12% SDS-polyacrylamide gel, electrophoresed and blotted to a PVDF membrane (Schleicher and Schuell, Keene, NH, USA). The membrane was blocked with 5% skim milk for 2 h, treated with goat polyclonal anti-mouse IgG overnight at 4°C, and then with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG antibody for 2 h at room temperature. Signal was detected using chemiluminescence reagent (DuPont NEN, Wilmington, DE, USA).

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SV40 mediates stable gene transfer in vivo

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Gene transfer in vivo requires an efficient, nonreplicating, transfer agent. We report here the efficacy of recombinant, replication-deficient SV40 in transferring firefly luciferase (luc) production to murine hematopoietic cells and selected internal organs in vivo. Replication-deficient SV40 was made by replacing the large T antigen gene (Tag) with a polylinker, into which luc cDNA (luc) was cloned. Luc expression was controlled by SV40 early promoter. Tag+ luc+ SV40 DNA was transfected into Tag-expressing cells to yield a replication-deficient SV40-derivative virus containing luc (SVluc). The ability of SVluc to transfer luc production in vivo was tested in two ways: SVluc was inoculated into BALB/C mice intravenously; also bone marrow cells treated with SVluc were infused into syngeneic hosts. Luc production was followed for 105 days by immunochemical analysis of peripheral blood and selected internal organs using anti-luciferase antibody, and by assay of luc enzyme activity in peripheral blood. Luc was found in 20–25% of peripheral blood nucleated cells from day 20 until ~105 days. Luc-producing cells were also identified in liver, spleen, brain, kidney, skin and colon from day 20 also until ~105 days. Analysis of whole blood showed fluctuating levels of functionally active luc enzyme beginning on day 21, and remaining substantially and significantly greater than control values to day 105. Thus, SV40 may transfer sustained expression of foreign genes to bone marrow and other organs, for at least 3 months.

Keywords: *gene therapy; viral vectors*

Introduction

Gene transfer to animals requires that transduced genes be expressed at sufficient levels, for sufficient time, to affect a target cell population. Most DNA transduction studies use viruses[1], or liposomes[2] as vehicles. Requirements of transfer agents vary, but usually include high infectivity, stability *in vivo*, and ability to avoid elimination by the immune system. Practicable gene transfer *in vivo* has proven difficult to achieve, largely because available delivery systems are not as effective as most clinical needs demand. Thus, the search for new gene transfer vectors continues.[3]

We report studies using a virus hitherto untested in gene transfer *in vivo*, simian virus-40 (SV40). SV40 is an attractive candidate for *in vivo* gene transfer for several reasons. It is a papovavirus with a circular double stranded DNA genome of approximately 5240 bp.[4] It can be concentrated to high infectivity. Wild-type (wt) SV40 usually does not complete a productive (lytic) infectious cycle except in its natural hosts, monkeys. However, it can infect cells of many tissues from diverse animal species, including man and rodents, and express its genes under the influence of its powerful early promoter (SV40-EP).[5]

SV40 DNA may integrate into the cellular genome, or be carried episomally. Integration occurs randomly, and genomic integration sites may not display characteristic flanking sequences as is the case for, eg retroviruses.[6,7] Activation of cellular genes at integration sites has not been associated with SV40.

The virus encodes a transforming protein, large T antigen (Tag). The role of Tag in SV40 replicative cycle and in host–virus interaction provides a handle by which to prepare a virus for gene transfer. Tag facilitates transformation of cultured cells by binding p53 and Rb tumor suppressor proteins.[8] However, despite transforming activity in culture when wt SV40 is inoculated in *vivo*, it causes tumors only in baby hamsters.[9,10]

Tag is also incorporated into the plasma membranes of infected cells, where it can be a target for host immune responses.[11,12] DNA replication and expression of late genes, including viral capsid genes, require Tag.[13,14] Removing *Tag*, then, should both mitigate immune responses to infected cells and render the virus replication-incompetent.

This report describes our studies of recombinant, Tag– SV40 as a gene-transfer agent.

Results

Generation of recombinant, replication-deficient SV40 containing luciferase (SVluc)

Production of SVluc is described in the Materials and methods section. Briefly, pBSV-1, which contains SV40 genome cloned into pBR322 (a kind gift of Dr JS Butel) was modified to create a shuttle vector, pBSV(luc) as shown in Figure 1. *Tag* was excised from pBSV-1 and replaced by a polylinker to create pBSV(ΔT). pBSV(ΔT) retains SV40 early promoter (SV40-EP) just upstream of the polylinker. Luciferase cDNA (*luc*), plus a second copy of SV40-EP, was cloned into pBSV(ΔT), to yield

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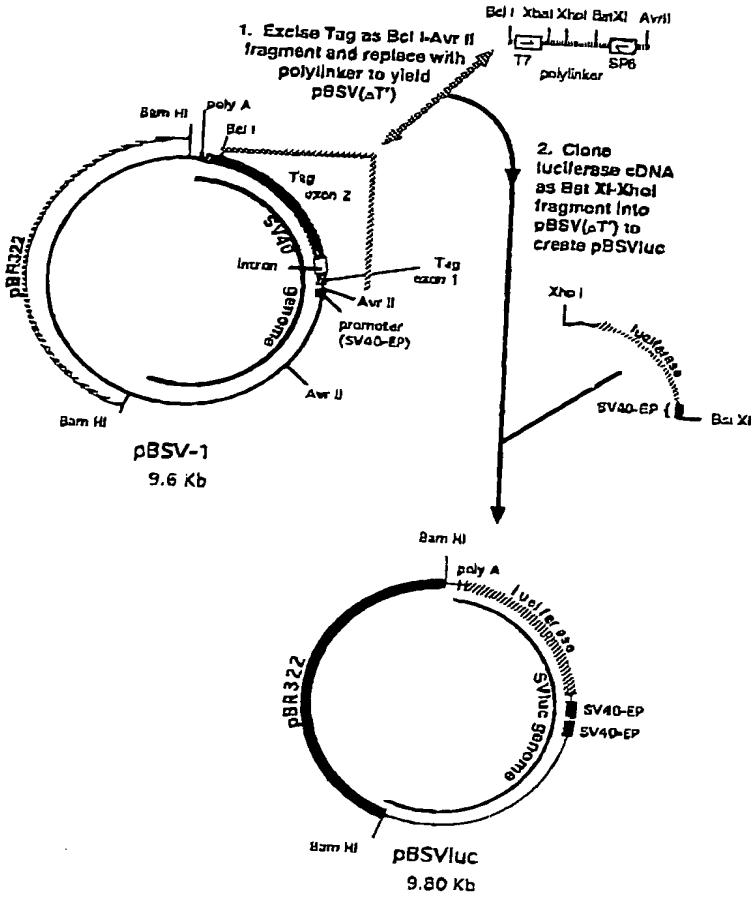

Figure 1 Production of pBSVluc. pBSV-1 (from Dr Janet S Butel, Baylor College of Medicine), containing the entire SV40 genome cloned as a BamHI fragment into pBR322, was digested partially with AvrII and completely with BclI, to excise the Tag gene. In its place, a polylinker with the indicated restriction sites, plus Sp6 and T7 promoters, was cloned as an AvrII-BclI fragment to yield pBSV(ΔT'). Luc, including an additional copy of SV40-EP, was cloned as a BstXI–XhoI fragment into compatible sites of pBSV(ΔT')

pBSV(luc). *Luc*-containing virus (SVluc) was made by excising the viral DNA from pBR322, recircularizing it, and using the circular *luc*-containing genome to transfect *Tag*+ COS-7 cells.

SVluc is replication-deficient

Tag is a necessary component of the virus replication complex. Therefore Tag is required for virus replication. If SVluc does not reacquire *Tag* during packaging, it will only replicate in cells that supply the lacking Tag in *trans* (eg COS-7 cells), but not in Tag– cells (eg TC7 cells). To ascertain whether SVluc was contaminated by virus that had become replication competent, we measured the ability of SVluc stocks to produce plaques in TC7 cells, compared to COS-7 cells (Table 1). SVluc produced plaques in COS-7 cells, our packaging cell line. In TC7 cells, however, SVluc failed to produce a single plaque, no matter how much SVluc was added to the cultures.

Table 1 SVluc is replication deficient

Virus added (p.f.u.)	*Plaques observed (COS-7 cells)*	*Plaques observed (TC7 cells)*
10^2	1.1×10^2	0
10^3	0.9×10^3	0
10^4	0.7×10^4	0
10^5	Confluent plaques	0
10^6	*NT*	0
10^7	*NT*	0

SVluc was titrated on COS-7 cells by standard serial dilution techniques, using neutral red-agar overlays. Based on those titers, doses from 10^2 to 10^6 p.f.u. SVluc were added to confluent cultures of COS-7 cells and TC7 cells. Plaque-forming units were measured in the same way.
NT: not tested

SVluc given I.v. transfers luciferase to multiple organs for at least 105 days

BALB/C mice received 10^7 p.f.u. SVluc i.v. Animals were killed approximately weekly from 3 to 7 weeks, and monthly thereafter. Frozen sections of colon, heart, liver, lungs, spleen, brain, stomach, tail skin (at the inoculation site) and diaphragm from SVluc recipients and control mice, were assayed for luc immunochemically (see Materials and methods). The most strongly positive tissue was skin at the inoculation site (Figure 2). Luc was detected consistently in >75% of local basal keratinocytes and also in suprabasal keratinocytes and occasional dermal fibroblasts, to 105 days post-inoculation (dpi).

To illustrate the specificity of this analysis, control tests on skin are also shown. SVluc recipient skin was stained using normal IgG instead of anti-luc antibody, and skin from control mice was stained with anti-luc antibody. Both were totally negative.

Luc was also detected in other tissues, as illustrated in Figure 3, and summarized in Table 2. Luc expression showed tissue and cellular tropisms. For example, epithelium of conducting airways (trachea, bronchi) was positive, but alveolar cells were consistently negative. Colon crypt epithelium was often positive, but stroma was not.

The percentage of positive cells did not change greatly for most tissues during these studies. Thus, in the liver, eg 1–2% of hepatocytes stained positively for luc from 20 to 105 dpi. In some organs, however, the population of positive cells did change over the course of the study. Splenic lymphocytes contained luc when assayed soon after inoculation (≤35 dpi), but beyond 47 dpi, only megakaryocytes stained positively for luc in the spleen. In some tissues, very few positive cells were noted: after i.v. inoculation luc was detected in the brain, but <0.1% of cortical neurons and glia stained positively for luc.

Histologic sections were studied carefully for evidence of inflammatory response, either mononuclear or polymorphonuclear, to luc-expressing (ie SVluc-infected) cells. At no time did we detect such a response in any tissue specimen examined.

Specimens of whole DNA from organs from SVluc recipients were analyzed to test whether SVluc integrated into the cellular genome or was carried episomally. Whole organ DNA was digested with *Not*I, which does not cut in SVluc, electrophoresed, transferred to nitrocellulose and hybridized with luc DNA. The only signal we visualized was in smears at molecular sizes >6 kb (not shown). SVluc genome is 5 kb.

Transfer of luc to hematopoietic stem cells

To test whether SV40 can effectively transfer gene expression to hematopoietic cells, BALB/cJ bone marrow was treated with SVluc *ex vivo* at MOI approximately 0.3, and infused into sublethally irradiated syngeneic mice (these animals are still alive). Effective transfer of luc to bone marrow stem cells was assayed in peripheral blood cells as luc production by immunochemistry, and as luc enzyme activity by luminometry.

Both mononuclear and polymorphonuclear leukocytes produced luc (Figure 3), ≥105 dpi. The percentages of each cell type bound by anti-luc antibody varied from one animal to another, but averaged 20 to 25% of peripheral blood nucleated cells (not shown). Platelets also reacted with anti-luc antiserum, while erythrocytes were generally negative.

Figure 2 Production of luc following SVluc infection. SVluc, 10^8 p.f.u., was inoculated i.v. into the tail vein of BALB/C mice. Skin at the inoculation site was frozen sectioned and treated with affinity-purified rabbit anti-luc antibody (Promega), using the ABC technique with horseradish peroxidase and diaminobenzidine, to identify cells producing luc.[16] The uppermost frame shows positive staining in basal and supra-basal keratinocytes of the tail skin of an SVluc recipient 105 dpi, denoting the presence of luc. The middle frame is the same area of the same tissue block of the same animal, treated with normal IgG instead of anti-luc antibody. It is negative. The lowest frame shows skin from a control (vehicle only) BALB/C mouse, treated with anti-luc antibody as for the first frame. It, too, is negative. Counterstained with hematoxylin, × 1100.

Luc enzymatic activity was measured by luminometry in these blood samples. Levels of luc function detected varied with time. However, luc activity was significantly ($P < 0.05$) and substantially (>10 ×) greater than control values obtained at most time-points, including 76 and 105 dpi (Figure 4).

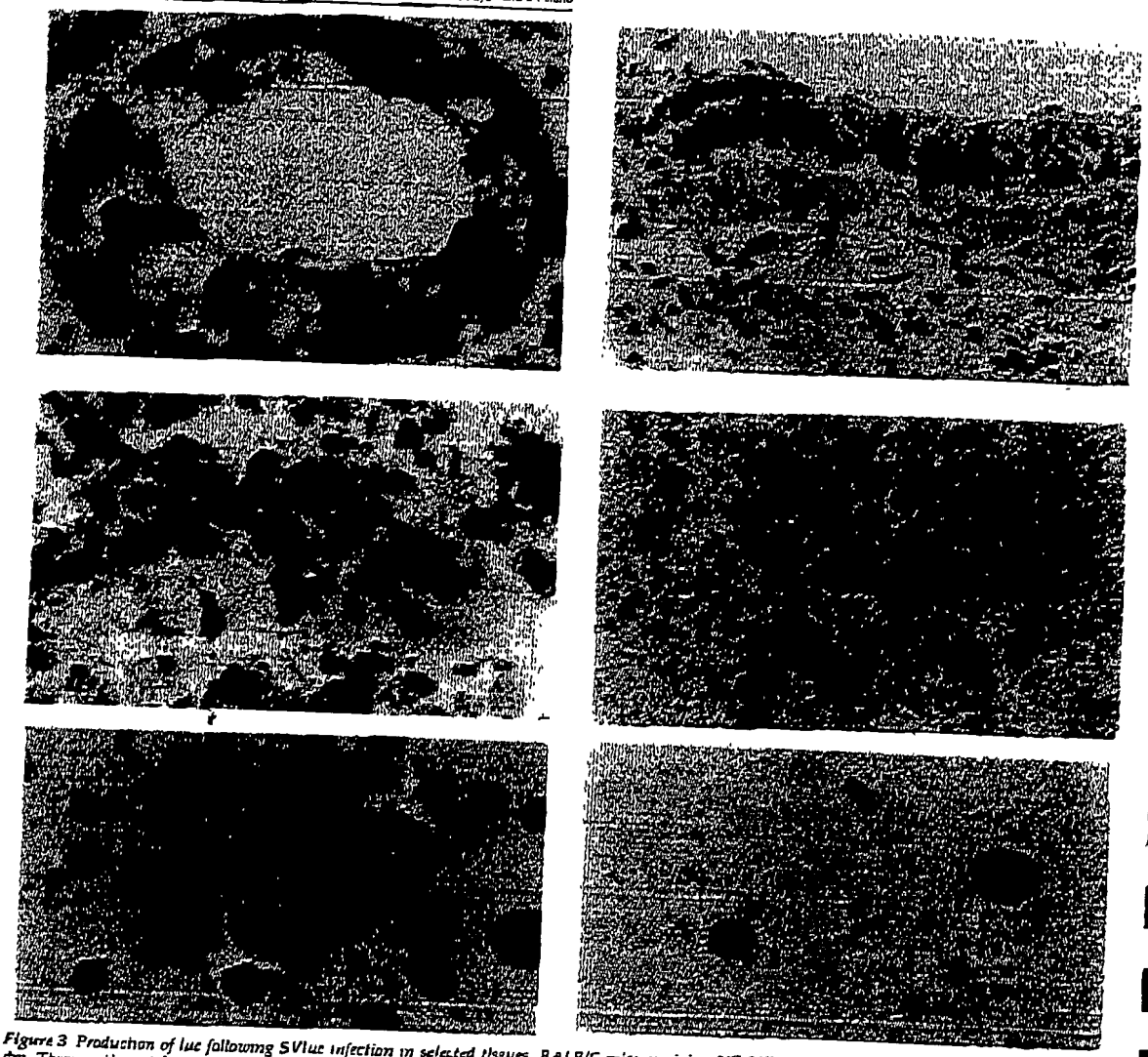

Figure 3 Production of luc following SVluc infection in selected tissues. BALB/C mice receiving 10^7 SVluc i.v. were killed at intervals from 20 to 105 dpi. These sections, taken from mice killed at the last time-point, demonstrate luc protein expression in cells of colonic epithelial crypts (upper left), and bronchus (upper right). The middle, left frame, shows two splenic megakaryocytes: the right one producing luc is adjacent to another megakaryocyte that is negative for luc. A cortical neuron and a subjacent glial cell are positive for luc (middle, right), as are hepatocytes (lower left). In addition, BALB/C mice receiving SVluc-treated syngeneic bone marrow cells were studied at similar time intervals for production of luc by peripheral blood cells. At 105 dpi, a representative peripheral blood smear from a recipient of SVluc-treated bone marrow was treated with anti-luc antibody, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (lower right). Positive staining, indicating the presence of luc, is visualized as red. In this field, a lymphocyte (left) and a neutrophil (right) both express luc. A small clump of platelets (upper middle) also express luc. Colon, spleen, liver, blood, × 2200. Bronchus, brain, × 1100.

Discussion

Several viruses are currently being used as vehicles for experimental gene transfer. Each has strengths and weaknesses.[15–17] Adenoviral vectors can be concentrated to high infectivity, express their genes in resting cells, and may carry large DNA inserts, but elicit strong immune responses that eliminate virus-infected cells within days.[18,19] Retroviral genomes integrate into host DNA and so may pass expression to daughter cells. However, retroviruses are difficult to concentrate, do not infect resting cells, and may be neutralized by serum components.[20,21] Adeno-associated virus is in the early stages of study, but it has also been difficult to produce in adequate concentrations.[22] Herpesviruses offer considerable promise for gene transfer to the central nervous

Table 2 Summary of tissue reactivity for luc in mice receiving SVluc IV

Tissue	Cellular localization	Degree of positivity	Days positive
Liver	Hepatocytes, Kupffer cells	-	20–105
Lung	Bronchial cells	+++	35–105
	Alveolar lining cells	-	
Colon	Crypt epithelium	+	20–105
Spleen	Lymphocytes	++	20–47
	Megakaryocytes	++	27–105
Brain	Neurons, glia	-	35–105
Skin	Basal and suprabasal keratinocytes	+++	20–105
Kidney	Glomerular and proximal tubular cells	+	20–105
Heart	None	-	
Diaphragm	None	-	
Stomach	None	-	

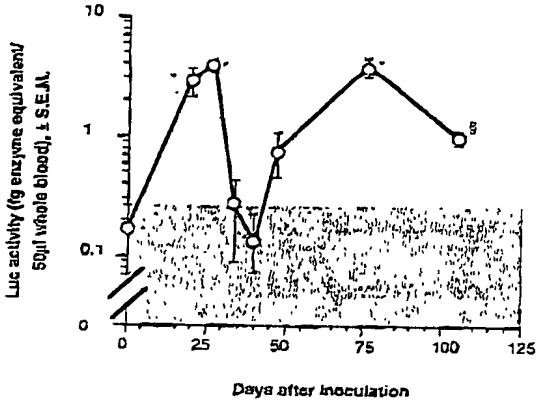

*Figure 4 Luciferase activity in the peripheral blood of mice receiving SVluc-treated bone marrow. BALB/C mouse recipients of SVluc-treated syngeneic bone marrow were bled periodically and luc activity of whole peripheral blood measured with a standardized assay (Promega). Emitted light values obtained from 50 µl of whole blood were compared to values obtained from (negative control) mice receiving untreated syngeneic bone marrow, and translated to a standardization curve of light-emission versus luc protein. Thus, emitted light, ± s.e.m., is expressed as fg equivalents/50 µl of whole blood. Each point is the mean of six independent determinations. The shaded area represents the fg equivalent values obtained from negative control samples. *P < 0.01 compared with control; §P < 0.05 compared with control.*

system,[23,24] but have not been extensively applied to other organ systems. Some of these viruses are being modified to circumvent their disadvantages,[25,26] but a considerable need for new and effective delivery systems remains.[27]

In theory, replication-deficient SV40 could circumvent many difficulties associated with other vectors for gene transfer *in vivo*. SV40 can be concentrated to high titer without losing infectivity. It can infect and express its genes in cells from many animal species and of diverse lineages. Its DNA may integrate into the host genome, thus permitting transduced gene expression in daughter cells. Removal of *Tag* creates space for inserted DNA, renders the virus replication-incompetent, eliminates an important viral antigen and prevents transcription of viral capsid proteins.

Taking advantage of these theoretical possibilities, we describe the first application of SV40 to gene transfer in animals. This approach takes cognizance of the fact that SV40 or fragments of SV40 DNA are often used in gene transfer *in vitro*. Plasmids incorporating parts of the SV40 genome, particularly SV40-EP and enhancer, are often used to transfer DNA to cells in culture. Also, there are several studies reporting use of replication-deficient SV40 to transfer DNA to cultured cells.[28,29]

We show here that SVluc transfers *luc* reporter gene activity *in vivo*. Luc protein and enzyme activity were demonstrable although SVluc was used at relatively low doses (10^7 p.f.u. per mouse i.v., and 10^9 p.f.u./3×10^7 bone marrow cells), and SV40-EP may not be the strongest promoter for *luc* expression.[30] Successful transduction was shown by immunologic detection of luc protein, and functionality demonstrated by enzyme assay.

For our immunochemical studies, the specificity of the staining reaction in tissue sections was ascertained using anti-luc antibody to test-stain tissues from control animals, and by using normal IgG on SVluc recipients' tissues. In both cases, no staining was seen. Luc was identified by immunochemistry in tissues from recipients of SVluc and in peripheral blood from recipients of SVluc-treated bone marrow. Cells from many organs produced luc, including hepatocytes, colonic and bronchial epithelia, neurons and glia, and hematopoietic precursor and mature peripheral blood cells. Other tissues, including heart and skeletal muscle, did not. We are currently testing whether differences in *luc* expression reflect tropism in SVluc infectivity, or tissue-specificity in gene expression or protein processing. Following transduction by SVluc, percentages of luc-producing cells were characteristic of tissue types and did not vary greatly from 20 to 105 days.

The luc produced by peripheral blood cells was functional. Significant luc enzymatic function was observed during the 105 day study period. Levels of luc activity fluctuated over this time, despite constancy in the percentage of peripheral blood nucleated cells positive for luc by immunochemistry (20–25%). These fluctuations appear to occur in parallel among experimental animals.

Since luc activity is temperature sensitive,[37] group variations in luc activity may have resulted from inconstancy in ambient temperature or sample handling, or fluctuations in hematopoiesis or leukocyte function. However, despite these fluctuations, statistically significant peripheral blood luc activity remained detectable over 3 months after bone marrow transfer.

Some gene-transfer agents, especially adenovirus, may elicit strong immune reactions that eliminate infected cells rapidly.[18,19] By contrast, luc production did not vary greatly during this study, and we found no evidence of immune elimination of, or inflammatory reaction to, luc-producing cells.

Because luc expression was strong in rapidly proliferating cells, eg peripheral blood nucleated cells, keratinocytes, etc it is likely that *luc* integrates into the host genome. Our data suggest that SVluc DNA is mostly integrated into the mouse genome within 14 dpi. Additional studies are in progress using both tissues from animals infected with SVluc and SVluc-infected cultured cells persistently producing luc.

Tag, the SV40 transforming protein, was excised from pBSV(ΔT'), our transfer plasmid, both to limit the potential immunogenicity of infected cells and to minimize the potential risks of applying this system therapeutically. It is possible for SVluc to reacquire Tag during passage in Tag+ cells. Reacquisition of Tag would restore replication competence. There are a number of ways to test for replication competence. The most sensitive technique is to compare viral plaque formation in permissive (COS-7) and nonpermissive (TC7) cells, both inoculated with known titers of virus. Plaque formation under agar requires virus replication, cell lysis, and spread of infectious virus to neighboring cells to repeat the process. In this way, we could detect a single replication competent virus contaminating a preparation of replication-incompetent virus.

By this approach, we did not observe any plaques in TC7 cells using as many as 10^6 p.f.u. SVluc. In these studies, the numbers of plaques observed in cultures of COS-7 receiving high doses of virus was somewhat lower than the actual number of p.f.u. added because of multiple infection. This finding was substantiated by our failure to detect late viral gene expression by Northern analysis (not shown). The consistent replication defect in SVluc suggests that reacquisition of Tag gene happens very rarely or not at all. Even were such a rare event to occur, however, analyses of Salk vaccine recipients show that even *wt* SV40, expressing *Tag*, is not demonstrably harmful to humans.[32,38]

Additional studies on this gene-transfer system are needed. *Luc* was similar in size (2.2 kb) to the *Tag* gene excised. The limitations SV40 imposes on insert size need to be defined. The host range of such SV40 constructs should be ascertained. SVluc infects human cells and stably expresses luc (L-X Duan, D Strayer, R Pomerantz, preliminary data), but the duration of expression and the nature of *luc* carriage in human cells are not yet known. We are also testing immune responses to SVluc to define mechanism(s) responsible for the apparent ability of SVluc-infected cells to escape elimination.

Thus, Tag– SV40 may effectively transduce sustained, functional gene expression *in vivo* to a number of tissue sites for at least 105 days. This system may be applicable to therapeutic gene transfer in clinical settings.

Materials and methods

Cell lines

We used two monkey cell lines: COS-7, which are Tag+ (ATCC) and TC7 (Tag–, a gift of Dr JS Butel). They were cultured in DMEM (GIBCO, Grand Island, NY, USA) + 10% serum.

Mice

Adult, female BALB/CJ mice (Jackson Laboratories, Bar Harbor, ME, USA) were used for all studies.

Plasmids

pBSV-1 (a gift of Dr Butel) contained wt SV40 genome cloned as a *Bam*HI fragment into pBR322. To make pBSV(luc), *Tag* was excised from pBSV-1 with *Bcl*I and *Avr*II, and replaced by a polylinker containing unique *Xba*I, *Xho*I and *Bst*XI sites, as well as Sp6 and T7 promoters (pBSV(ΔT')). This construct retains SV40-EP just upstream of the polylinker. Firefly luciferase cDNA (*luc*), plus a second copy of SV40-EP were cloned into pBSV(ΔT'), to yield pBSV(luc).

SVluc

Luc-containing virus, SVluc, was made from pBSV(luc) by excising the viral genome with *Bam*HI. It was gel purified, recircularized, and transfected into COS-7 cells.[34] Virus was harvested, purified and titrated according to standard methods.[39]

The inability of SVluc to replicate in cells that do not supply Tag was tested by adding known amounts of SVluc to cultures of COS-7 cells or TC7 cells, and measuring virus p.f.u. using standard techniques.[39]

Virus inoculation

SVluc was given to BALB/CJ mice via one of two routes: (1) 10^7 plaque-forming units (p.f.u.) were administered i.v. in 0.1 ml saline (control mice received vehicle alone); or (2) femoral bone marrow from BALB/CJ mice was treated with SVluc (MOI approximately 0.3) for 6 h before transfer. Bone marrow recipients received 450R external beam radiation from a Cs source (Division of Radiation Therapy, Jefferson Medical College), followed by the SVluc-treated bone marrow, 3×10^7 nucleated cells/mouse, i.v. Control mice received untreated marrow.

Tissue preparation

Peripheral blood was sampled from bone marrow recipients at intervals. It was kept frozen at –70°C for luciferase assays. Smears were air-dried, and fixed in acetone. Recipients of SVluc i.v. were killed from 20 to 105 dpi. Selected tissues (stomach, lung, liver, spleen, kidney, heart, brain, colon, skin at the inoculation site and diaphragm) were excised and frozen for sectioning.

Analysis of DNA persistence was determined by homogenizing tissues, extracting DNA, and digesting this DNA using an enzyme that does not cut in the SVluc genome, *Not*I. The digested DNA was then electrophoresed, blotted to nitrocellulose and probed with luciferase DNA. DNA fragments that hybridized with the probe were visualized by autoradiography.

Luciferase analysis

Luc was detected enzymatically in whole peripheral blood using Luciferase detection kit (Promega, Madison,

WI, USA) according to package instructions. Luc-producing cells were visualized in peripheral blood smears or frozen tissue sections by immunostaining.[36] Affinity-purified anti-luc antibody (Promega, diluted 1:1600), or normal IgG, was applied, followed by biotin-goat anti-rabbit IgG, then avidin–peroxidase (tissue sections) or avidin–alkaline phosphatase (peripheral blood) then substrate.[36] As negative controls, identical serial sections, stained with normal IgG, and tissues from control mice were stained using the anti-luc antibody. Luc production was evaluated semiquantitatively, − to ++++, based on the number of positive cells and strength of staining, relative to negative controls.

Acknowledgements

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Note added in proof

Subsequent assay of SVluc recipients revealed continued expression of luciferase at 6 months following receipt of virus or of virus-treated bone marrow cells. Levels of expression at 6 months were comparable to those seen at 3 months.

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A novel SV40-based vector successfully transduces and expresses an alpha 1-antitrypsin ribozyme in a human hepatoma-derived cell line

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Alpha 1-antitrypsin (α1AT) deficiency disease is one of the more common hereditary disorders that affects the liver and lung. The liver disease of α1AT deficiency is generally thought to be caused by the accumulation of an abnormal α1AT protein in hepatocytes, whereas the lung disease is thought to be due to a relative lack of the normal protein in the circulation. Therefore, one possible approach to prevent and treat α1AT disease is to both inhibit the expression of the mutated α1AT gene, and to provide a means of synthesizing the normal protein. To do this, we designed specific hammerhead ribozymes that were capable of cleaving the α1AT mRNA at specific sites, and constructed a modified α1AT cDNA not susceptible to ribozyme cleavage. Ribozymes were effective in inhibiting α1AT expression in a human hepatoma cell line using a newly developed simian virus (SV40) vector system. In addition, the hepatoma cell line was stably transduced with a modified α1AT cDNA that was capable of producing wild-type α1AT protein, but was not cleaved by the ribozyme that decreased endogenous α1AT expression. These results suggest that ribozymes can be employed for the specific inhibition of an abnormal α1AT gene product, the first step in designing a gene therapy for the disease. The findings also suggest that the novel SV40-derived vector may represent a fundamental improvement in the gene therapeutic armamentarium.

Keywords: *ribozymes; SV40; gene therapy; α1-antitrypsin deficiency*

Introduction

Alpha 1-antitrypsin (α1AT) deficiency, one of the more common lethal hereditary disorders in Caucasians of European descent, is characterized by reducing serum levels of α1AT, a 52-kDa glycoprotein that functions as an antiprotease.[1] The deficiency state is caused by mutations of the α1AT gene, a pleiomorphic, 12.2-kb 7-exon gene.[2] Normal α1AT serum levels are 20–53 μM;[3] various combinations of at least 17 different mutations of the α1AT gene are associated with an α1AT level <11 μM and significant risk for developing emphysema.[4] A subset of mutations is associated with hepatitis and cirrhosis.[5] These latter mutations all involve the production of abnormal proteins: they do not include null mutations. The pathogenesis of the liver disease is thought to be due to the accumulation of an abnormal α1AT protein in hepatocytes, and is associated with the finding that certain mutations of the α1AT gene cause derangement in the protein's intracellular processing and defects in the protein's excretion, commonly associated with liver injury.[6] The molecular defect in the protease inhibitor (Pi)Z allele, the allele most commonly associated with liver injury, is a G to A transition resulting in a Glu to Lys substitution at amino acid 342. This mutation is thought to cause the variant protein to aggregate in the rough endoplasmic reticulum of the liver cells.[4,6]

Although several studies have focused on the delivery of the normal α1AT gene into hepatocytes or airway cells to restore normal α1AT production and to protect lung tissue,[7–9] these approaches do not affect the liver disease. It would appear that the best way to treat α1AT deficiency disease is to reduce the production of the endogenous mutant form of α1AT protein and to increase the synthesis of the normal protein. The reduction of α1AT expression can be achieved by several strategies, such as antisense, gene-specific ribozymes, α1AT transcription factor specific-inhibitors, or intracellular expression of antibody to the mutant form of the α1AT protein.

Recently, ribozymes have been targeted to a wide variety of substrates and tested in biological systems to achieve the inhibition of cellular gene expression or viral replication.[10–15] Ribozymes have an advantage in the therapy of chronic disease since they are not degraded when the target RNA is cleaved, and therefore, theoretically they need not be produced at higher levels than the target transcript. By using a hammerhead ribozyme, we should be able to target the mutant form of α1AT mRNA by using α1AT guide sequences attached to the ribozyme catalytic core sequence. Furthermore, if we change the corresponding cloned wild-type α1AT cDNA GUC and guide region nucleotide sequences while maintaining the amino acid sequence, this modified 'wild-type' α1AT mRNA should be resistant to the ribozyme.

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A major issue in designing adequate gene therapeutic approaches to diseases is the availability of an effective vector system that will both deliver the gene of interest to the target organ and allow for its adequate expression. One of our laboratories has recently engineered the simain virus (SV)40 so that it has become an effective transfer vector system.[16,17] In the present study, we report the transduction of an effective α1AT ribozyme in this SV40 vector system to inhibit the expression of the α1AT gene in a human hepatoma-derived cell line. At the same time, we have constructed a modified α1AT cDNA that is capable of producing the normal α1AT protein, yet its RNA is resistant to ribozyme cleavage.

Results

To study the effects of ribozymes on the expression of α1AT in a human hepatoma-derived cell line, we transduced PLC/PRF/5 cells that actively produce α1AT with either retroviral or SV40 recombinant viruses. Both polymerase II and III promoters were employed to drive expression of the ribozymes.

In one series of studies,[14] retroviral vectors were used to transfer ribozymes. RNA was extracted from a pooled population of cells that had been tranduced with the retroviral vectors and selected with G418 for 3 weeks. Those studies indicated that whereas ribozyme expression driven by a tRNA promoter decreased α1AT mRNA expression by as much as 85%, no such effect on α1AT mRNA expression was found when the CMV promoter was used to drive ribozyme expression. The SV40 vector system was somewhat more effective in that ribozymes driven by a polymerase II promoter were effective when transduced with the new vector system. PLC/PRF/5 cells were transduced using three different SV40 constructs, each containing either AT204 or 589 ribozyme. One construct employed the AT204 ribozyme driven by the tRNA promoter, one used the AT589 ribozyme under the control of the SV40 early promoter, and the third used the AT589 ribozyme with the tRNA promoter. No selection was applied to these cells. Forty-eight hours following transduction, RNA was isolated from the unselected populations of cells and assayed by Northern blot hybridization analysis. α1AT mRNA levels were significantly decreased in cells infected with the recombinant viruses in which ribozyme expression were driven by either the SV40 early promoter or the tRNA promoter, whereas there was no difference in GAPDH expression (Figure 1)

Further studies were done with ribozyme AT589 constructs because they generated consistently high ribozyme activity. Figure 2 is a series of representative Northern blots of RNA isolated from an unselected population of cells 48 h following transduction with a control construct, SVCAT, or with vector constructs containing 589AT ribozymes. The blots demonstrate the effectiveness of the ribozymes and their selective and specific properties.

In addition, we employed the AT589 construct in experiments of longer duration to demonstrate the effectiveness of the construct over time. The PLC/PRF/5 cells were transduced with the SV(Δ).tAT589 construct, then RNA was extracted at days 2, 7 and 14 following transduction. Figure 3 demonstrates that the ribozyme effectively cleaved the α1AT mRNA after 2 weeks of infection

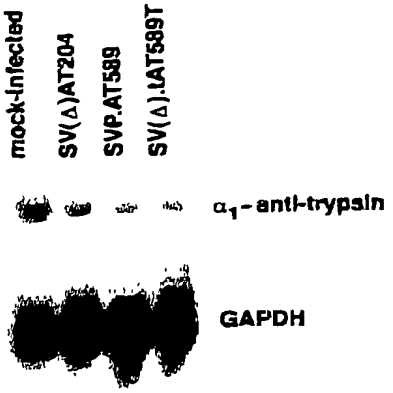


Figure 1 Representative Northern blot of RNA extracted from an unselected population of PLC/PRF/5 cells that were mock-infected or infected 48 h previously with an SV40-derived vector containing an α1AT ribozyme driven by either the SV40 early promoter (SVP.AT589) or by a tRNA promoter [SV(Δ)AT204] or [SV(Δ).tAT589T] at an MOI of 10. The RNA was electrophoresed, then hybridized with α1AT or GAPDH probes, as described in Materials and methods.

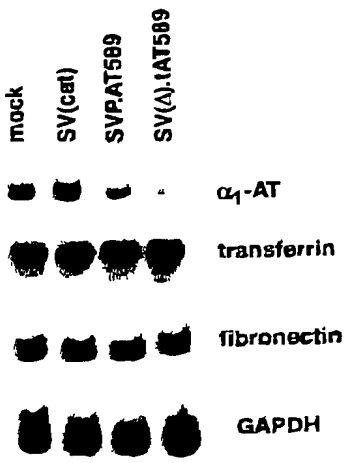


Figure 2 Northern blot hybridization analysis of α1AT ribozymes employing an SV-40 vector system. PLC/PRF/5 cells were infected in culture, with SV(Δ)CAT, SVP AT589, or SV(Δ).tAT589 at MOI of approximately 100, or they were mock-infected. Cells were infected and RNA extracted as in Materials and methods. SV(Δ)CAT represents a control for transduction

despite the cells having a doubling time of 3 days. This suggests that the construct was integrated, although no proof of integration was demonstrated. Another alternative explanation is that the high MOI employed allows for episomal expression in daughter cells.

To compensate for the endogenous α1AT expression being reduced by the AT589 ribozyme, we modified the nucleonde sequences of the target site of AT589 in the full-length α1AT cDNA, without changing amino acid

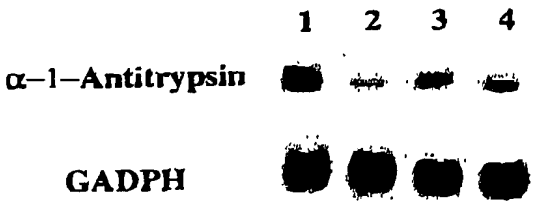


Figure 3 Northern blot hybridization analysis of α1AT and GAPDH mRNA in a PLC/PRF/5 cell population mock-infected or infected with an SV40-derived vector containing the AT589 ribozyme Cells were transduced with the SV(Δ) tAT589T viral construct for different times or were mock-infected; then mRNA was extracted and Northern blot hybridization undertaken as described in Materials and methods. Lane 1, mock-infected cells; 2, cells harvested 2 days after infection; 3, cells harvested 7 days after infection; 4, cells harvested 14 days after infection.

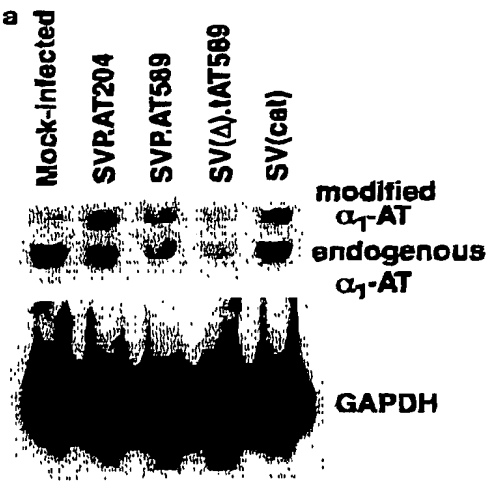
sequences, to create the modified α1AT cDNA that would be resistant to AT589 ribozyme cleavage. To test the effectiveness of this construct, we stably transfected the PLC/PRF/5 cell line with the modified α1AT cDNA construct in a retroviral vector under the control of the CMV promoter. When we then transduced this line with the SV40 vectors containing ribozymes, the endogenous α1AT mRNA expression was considerably decreased by the two AT589 ribozyme constructs. The 204 construct had little if any effect. There was no effect of the ribozymes on the modified α1AT mRNA (Figure 4a). Transduction with a control construct, SV(Δ)CAT, perhaps slightly decreased the expression of the endogenous α1AT mRNA in this experiment, but not in other experiments (see Figure 2). The specific nature of the ribozymes was demonstrated again when GAPDH was employed as a control; no effect on GAPDH expression was found with any ribozyme. Densitometry scanning of the experiments confirmed that the 589AT ribozymes were effective in selectively affecting the endogenous α1AT, without having an effect on the modified species (Figure 4b).

Densitometry scanning of three sets of experiments indicated that the SVP.AT589 construct (driven by the SV-40 early promoter) decreased α1AT expression by 70.8 ± 4.0% (mean ± s.e.m.). The SV(Δ).tAT589 construct (driven by the tRNA promoter) decreased α1AT expression by 74.8 ± 2.1%.

To demonstrate that the modified cDNA is capable of producing a wild-type protein, two cell lines (HeLa and NIH 3T3) that do not express any endogenous α1AT protein were transduced with an SV40-derived vector that contained the modified α1AT cDNA. Figure 5 is a Western blot that shows that the transduced cell lines produce a quantitatively and qualitatively similar α1AT protein product when compared to the wild-type protein in the PLC/PRF/5 cell line.

Discussion

The present study indicates that hammerhead ribozymes can be effectively used to inhibit expression of α1AT in a hepatoma-derived cell line. The inhibitory activity of the ribozymes seems to depend on the ability to provide a high level of ribozyme expression. Our findings, in these and other studies, demonstrate that the ribozymes



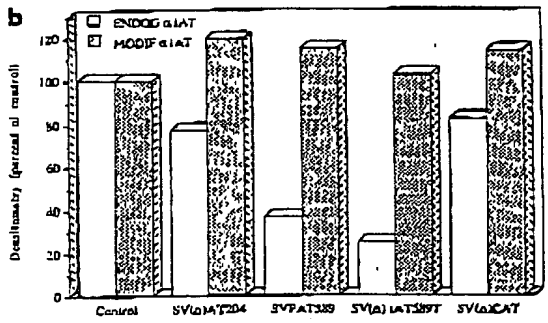


Figure 4 (a) Northern blot hybridization analysis of the effects of α1AT ribozymes on the expression of endogenous and modified α1AT in PLC/PRF5 cells. The hepatoma cell line was stably tranduced with pSLXCMVmα1AT which lead to expression of the modified α1AT, then transduced with SV-40 virus at an MOI of approximately 100. Lane 1, untransduced cells; 2, cells transduced with SVP.AT204; 3, transduced with SVP.AT589; 4, SV(Δ) tAT589T; SV(Δ)CAT, in lane 5, represents a control for transduction. Pooled cells from whole cultures were used and RNA extracted 48 h after the second transduction. (b) Densitometry tracing of (a) showing effect of ribozymes on endogenous and modified α1AT levels

were effective whether they were transduced using the standard retroviral vector system employing a selected population of stably transfected cells, or employing the novel SV40 vector system without selecting for transduced cells. In addition, our results show that it is possible to transduce a modified cDNA into hepatoma cells, and that the transcript which encodes for the wild-type α1AT protein is not susceptible to ribozyme cleavage. These results suggest that the use of a 'bifunctional' vector may be a possible approach for the prevention and treatment of α1AT deficiency.

Although ribozymes are now recognized as a powerful extension of the antisense approach to gene therapy by their ability to selectively cleave specific mRNA species,[10–13] most previous reports employing ribozymes

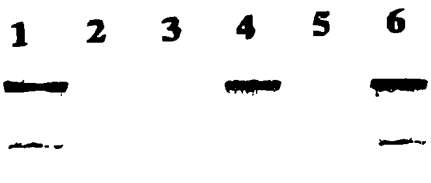


Figure 5 Western blot hybridization analysis of cells mock-infected or infected with an SV40-derived vector containing a modified α1AT cDNA. Cells were transduced with the SVCMVmα1AT vector or mock-infected, then 2 days later protein was extracted and Western blot analysis undertaken with a human α1AT polyclonal antibody as described in Materials and methods. Lane 1, PLC/PRF/5 cells mock-infected; 2, molecular weight marker; 3, NIH 3T3 fibroblast cells mock-infected; 4, NIH 3T3 cells infected with the SVCMVmα1AT construct; 5, HeLa cells mock-infected; 6, HeLa cells infected with the SVCMVmα1AT construct.

showed their successful targeting only in single cell clones.[11–14] In addition, most reports involve targeting viral transcripts, levels of whose expression may be affected by controlling the virus:cell ratio.[14–22] There are very few reports that demonstrated adequate inhibition of an endogenous cellular transcript – especially one expressed at high levels, and especially in a mixed population of transduced cells.[10,15] Two recent reports show the effectiveness of ribozymes in inhibiting the expression of the transgene in transgenic mice.[23,24] The common thread in most of these reports is the necessity for ribozymes to be expressed at high levels in order to inhibit the target gene expression.

In our studies, we determined that a tRNA promoter was required for the adequate expression of the ribozyme when the retroviral vector was employed; α1AT expression was not inhibited when the CMV promoter was used. This most likely occurred because the tRNA promoter is probably the best promoter for transcribing short RNA species.[14,15,25,26] Although we do not demonstrate in this report that the tRNA promoter-driven constructs synthesized greater amounts of the ribozymes, that is our speculation. Another possible explanation is the difference between the polymerase II and III transcripts. Recently it was shown that modifications of the 3′ end of ribozymes dramatically affected the intracellular stability and cleavage efficiency.[25] The RNAs transcribed by RNA polymerase II have long poly (A) tails which may affect the structure of the ribozyme and subsequently disturb the effective association with target RNA and the cleavage reaction. In addition, retroviral transcripts are often generated from long terminal repeat (LTR) to LTR,[27,28] providing very bulky flanking RNA sequences that may interfere with catalytic and/or binding activation.

It appears that in some ways the newly described SV40 vector system[16,17] may be more efficient in transducing and expressing its transgene than the retroviral system. We were able to inhibit α1AT expression with recombinant SV40 employing either the SV40 early promoter or the tRNA promoter. What makes the finding more impressive is that the effect on α1AT expression occurred in an unselected population of cells. This contrasts with the experience with the retroviral system, where cells were selected by G418 resistance, and only successfully transduced cells were evaluated. Such selection was not used in the SV40 experiments; RNA was isolated from the entire population of cells 48 h after infection with recombinant SV40 vector. No attempt was made to select a subpopulation of transduced cells. Moreover, the SV40-derived vector was successful in inhibiting α1AT expression 2 weeks following transduction, suggesting an integration event in these rapidly proliferating cells.

The SV40 system appears to have significant potential as a vector for gene therapy. This expression system, which is described in greater detail in earlier reports,[16,17] uses to advantage the ability of SV40 to express encoded genes in many cell types and in different animals, its ability to infect and to drive expression in non-dividing cells, the lack of any pathologic immune response evoked by transduced cells, the relatively small size of the viral genome, the availability of cell lines that express various portions of the SV40 genome, and the strong SV40 early promoter. This system has been remarkably successful in animal experiments in mediating stable, strong expression of a reporter gene whose product can be detected by both enzymatic and immunohistochemical means. Firefly luciferase was cloned into the SV40 vector to produce a recombinant virus, SVluc. This recombinant virus was used to infect cultured target cells in which luciferase was detected immunohistochemically.[16] SVluc was also used to infect BALB/c mice *in vivo*.[17] Following intravenous inoculation, stable luciferase activity was detected by immunohistochemical analysis of frozen section tissues and by luminometric assay. Levels of enzyme expression in liver and other organs were stable for the duration of the experiment, 1 year,[17] without evidence of inflammatory reaction to the luciferase-expressing cells.

In the present report, we have delineated the first steps in the inhibition of abnormal α1AT gene expression, as well as the synthesis of a normal gene product for the treatment of α1AT deficiency disease. It appears that SV40 provides a promising new vector technology for designing feasible gene therapy systems. Further experiments must show the ability of the system to function in an adequate *in vivo* model.

Materials and methods

Ribozyme design and cloning

The details of the construct and screening of functional α1AT ribozymes have been reported by us previously.[26] The ribozymes that target α1AT mRNA at 204 and 589 were selected for testing in the SV40-derived system. Briefly, each ribozyme is denoted by the nucleotide position of the first G of the target GUC triplet when the first A of the initiation codon AUG is numbered as 1. The sequences of the ribozymes are shown in Figure 6. For the design of the ribozymes, 11 to 15 bases of antisence sequence against α1AT mRNA were flanked on both sides of the hammerhead motif to allow the ribozyme to associate with α1AT mRNA through their complementary sequences. For the construction of the ribozymes, two complementary oligonucleotides were synthesized on the DNA synthesizer model 392 (Applied Biosystems, Foster City, CA, USA). The ribozymes were synthesized

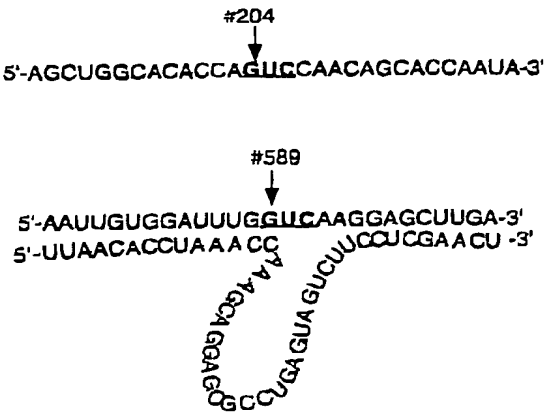


Figure 6 Hammerhead ribozymes that were most successful in cleaving α1AT mRNA in cell-free systems. Antisense sequences against each region of the α1AT mRNA were employed on both sides of the hammerhead motif.

by incubating two oligonucleotides to form a hemiduplex, and PCR amplifications were performed. Then the PCR products were cloned directly into the pT7Blue-T vector (Novagen, Madison, WI, USA) to generate plasmids pT7ATRzs, which contained each ribozyme under the control of the bacteriophage T7 promoter.

Construction of modified α1AT cDNA

The details of the construction of a modified α1AT cDNA which would be resistant to an α1AT ribozyme and yet code for normal α1AT protein, have been reported by us previously.[18] This modified cDNA has the third nucleotide mutated in the 589 region (GTC — GTG), and in the immediate 5' and 3' flanking region. It is shown in Figure 7. Briefly, the full-length α1AT cDNA was cloned into pT7 Blue-T vector (Novagen) to generate pT7α1AT by RT-PCR, employing RNA from Hep G2 cells. Then modified oligonucleotides were used as templates for the PCR reaction leading to the generation of the modified α1AT. The PCR products were then cloned into the pT7Blue-T vector, to generate pT7mα1AT.

Retroviral vector

For construction of the retroviral construct which carries a modified α1AT cDNA, the 1.3 kb fragment of human full-length, modified α1AT cDNA was excised from pT7mα1AT with *Xba*I and *Sma*I, and recloned into the *Xba*I–*Pvu*II site of the pSP72 vector (Promega) to generate pSP72α1AT. The modified α1AT cDNA was cut with *Bgl*II and *Xho*I, and cloned into the *Bam*HI–*Bgl*II site of pSLXCMV[50] to generate pSLXCMVmα1AT (Figure 8).

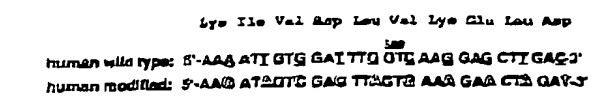


Figure 7 The sequences of the modified cDNA with the third nucleotide mutated in the region of the ribozyme recognition signal at nucleotide position 589.

Figure 8 Structure of the retroviral vector that expresses the modified α1AT cDNA. Arrow shows the direction of transcription.

Orientations and sequences of retroviral vectors were confirmed by DNA sequencing.

Cells and transfection

The human hepatoma-derived cell line PLC/PRF/5 and the retrovirus packaging cell line PA317 were grown in DMEM supplemented with 10% fetal bovine serum. Subconfluent PA317 cells were transfected with plasmid pSLXCMVMα1AT by lipofectin (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's instruction manual. After 48 h, medium containing recombinant retrovirus particles was collected, and PLC/PRF/5 cells were transduced with the recombinant virus for 48 h; then PLC/PRF/5 cells were selected with 1 mg/ml of G418 (Bibco BRL) for 3 weeks. G418-resistant clones were picked under microscopy and subjected to further analysis.

Construction of SV40-derivative viruses for transduction

The details of the construction and application of modified SV40 vectors for the purpose of gene transduction have been reported elsewhere by us.[15,17] The procedure is described briefly here. The 5.24 kb SV40 genome, cloned as a *Bam*HI fragment into pBR322, was the kind gift of Dr Janet Butel (Baylor College of Medicine). The viral genome was modified by excising the gene for large T antigen (Tag) as an *Avr*II–*Bcl*I fragment, and replacing it by a modified pGEM7 polylinker, whose Sp6 and T7 promoters were flanked by *Avr*II and *Bcl*I restriction sites, respectively. This procedure also excises the gene for the small T antigen, but leaves the SV40 early promoter and the SV40 polyadenylation signal intact. The late viral genes, VP1, VP2 and VP3, which encode capsid proteins, are also undisturbed in this construct. This modified SV40 genome, still in pBR322, was named pBSV(ΔT').

To produce virus from this construct, the viral genome was excised from pBR322, gel purified and recircularized. It was then transfected into COS-7 cells (ATCC) for packaging. The COS-7 cells used for this procedure contain a copy of the SV40 genome that is deficient in that it is a deletion in the origin of replication. Thus, these cells supply the missing Tag *in trans* and permit the replication and packaging of the resultant Tag-deleted viruses. TC7 cells (a kind gift of Dr J Butel), lacking the viral genome, do not permit replication of Tag-deleted SV40 derivatives.[17] This system therefore does not depend upon the presence of helper virus, but relies on expression of Tag by the packaging cells.

After visual examination for virus-induced cytopathic effect showed that >50% of COS-7 cells have been infected with virus, virus was released from the cells by freezing and thawing, followed by sonication. Most of the infectious virus remained associated with the cell cytoskeleton. Virus was titrated by measuring the concentration of infectious units that can penetrate target cells

by an *in situ* polymerase chain reaction.[31] In general, yields of infectious virus without further purification are 10^9 $TCID_{50}$/ml.

Concentration of virus to approximately 10^{10} $TCID_{50}$/ml was accomplished by discontinuous sucrose density ultracentrifugation. This procedure was performed according to the method of Rosenberg *et al*.[32] Briefly, culture medium containing the SV40 derivative viruses was mixed 1:10 with a solution of 10% Triton X-100 + 5% deoxycholate, and layered on to a discontinuous sucrose density gradient (20% sucrose/75% sucrose) and centrifuged at 65 000 *g* in an SW28 rotor in a Beckman ultracentrifuge. This procedure dissociates virus from the associated COS cell cytoskeleton. Virus concentrated at the interface between the two sucrose layers is recovered, then dialyzed *versus* normal saline to remove excess sucrose. These preparations are titrated before use.[31]

The ribozyme constructs described above were cloned into the multiple cloning site of pBSV(ΔT). These cloning procedures yielded two different types of ribozyme-containing plasmids: one in which ribozyme expression was driven by the SV40 early promoter, and another in which the tRNA promoter was used to drive ribozyme transcription. Replication-incompetent SV40 derivative viruses containing these ribozyme constructs were produced and titrated as described above. These vectors are shown in Figure 9.

Recombinant viruses, containing the ribozymes under the control of either the tRNA or SV40 early promoter, were used to transduce PLC/PRF/5 cells at an MOI of either 10 or 100. In one experiment, the hepatoma cells were stably transduced with the modified α1AT cDNA, by the use of vector pSLXCMVmα1AT before transduction with the SV40-derived vectors. Two days following the transduction, RNA was isolated from an unselected population of cells and evaluated by Northern blot hybridization analysis.

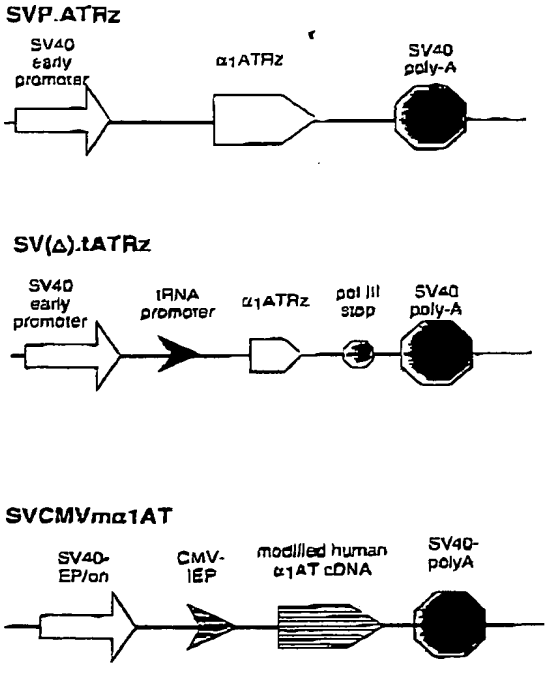


Figure 9 Structures of the SV40-derived vectors that express the α1AT ribozymes or the modified α1AT cDNA.

RNA analysis

Total RNA from PLC/PRF/5 cells transduced or non-transduced with viral constructs was extracted by a modification of the method of Chomczynski and Sacchi.[33] Messenger RNAs were detected by Northern hybridization analysis as previously described[34] employing a human α1AT cDNA probe; human fibronectin, human transferrin and glyceraldehyde dehydrogenase (GAPDH)[35] were employed as controls. Samples of 20 μg of total RNA were denatured in buffer containing 0.5 mg/l glyoxal, 50% dimethyl sulfoxide, 10 mM phosphate, electrophoresced in 1% agarose gel, transferred to a GeneScreen filter (New England Nuclear, Boston, MA, USA), and baked for 2 h at 80°C. The filters were prehybridized and were subsequently hybridized under stringent conditions with cDNAs labeled with α-^{32}P-dCTP by a primer extension kit (Amersham, Arlington Heights, IL, USA). After hybridization, the filters were washed and the signals were visualized by autoradiography. Densitometry analysis of the membranes was undertaken with the use of a phosphorimager (Molecular Dynamics).

Western blot analysis

HeLa and NIH 3T3 cells were cultured in DMEM plus fetal calf serum. Then each cell line was infected with an SV40 viral construct containing the modified α1AT cDNA, SVCMVmα1AT. Forty-eight hours following infection the unselected cells were harvested and protein extracted as previously described. Proteins were subjected to electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gels and subsequently transferred to a Poly Screen (PVDF) membrane (Dupont). After the membrane was blocked with 5% non-fat dry milk, the specific protein expression was detected with rabbit polyclonal anti-human α1AT antibodies (Boehringer Mannheim, Indianapolis, IN, USA), using the Dupont Western blot chemiluminescence kit, with the manufacturer's suggested protocol.

Acknowledgements

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Use of SV40 to immunize against hepatitis B surface antigen: implications for the use of SV40 for gene transduction and its use as an immunizing agent

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We have described a novel gene transfer system, in which replication-incompetent T antigen-deleted simian virus 40 (SV40) is used as the transduction vehicle. We report here successful immunization using such an SV40-derived viral vector. Hepatitis B surface antigen (HBsAg) cDNA was cloned downstream of two tandem SV40 early promoters to yield a T antigen-deficient SV40-derivative, SV(HBS). Cultured TC7 cells were exposed to SV(HBS), and expression of HBsAg was detected 24 h later by Northern blot and RT-PCR analysis. Immunochemistry and Western blot analysis were also performed 24 h after infection to detect expression of HBsAg. Once it was ascertained that we could express HBsAg in this way, we used SV(HBS) to elicit anti-HBs. SV(HBS) was injected intraperitoneally or subcutaneously into mice every 4 weeks. These mice were bled every 2 weeks and their sera assayed for antibody activity against HBsAg and SV40. Production of anti-HBs was measured by ELISA and confirmed by Western blot analysis, both of which demonstrated significant levels of anti-HBs after the second injection. We also tested production of anti-SV40 antibodies by the ability of sera to neutralize SV(HBS) infectivity. We found no evidence of neutralization of SV(HBS) infectivity even after eight inoculations. Thus, replication-incompetent SV40 is still not a strong antigen. Our data suggest that SV40-based transduction systems may be useful vehicle for immunization and for other gene transfer applications when a need for multiple inoculations is anticipated.

Keywords: gene transfer; papovavirus; transgene expression

Introduction

We have reported a novel gene transfer system, in which SV40 is used as a transduction vehicle.[1–5] SV40 infects resting as well as cycling cells, and is not known to be inactivated by human complement. Its DNA may integrate into the genome of target cells, or it may express its genes episomally, usually as a minichromosome.[4] We have found that SV40 can transduce firefly luciferase (luc) activity stably for >12 months into murine bone marrow cells *in vivo*.[3] We have also shown that SV40-derived vectors can transduce resting human peripheral blood mononuclear cells (PBMC) using two different reporter genes, HBsAg and luc.[5]

Hepatitis B virus (HBV) infection is endemic to many parts of the world and is one of the most important chronic virus infections, with over 300 million HBV carriers worldwide.[6] Infection is transmitted most commonly from chronically infected mothers to their newborns, who consequently often develop chronic hepatitis B. Many adults exposed to HBV suffer acute infections, a minority of which do not resolve but rather progress to chronic hepatitis B.[7] Long-term sequelae of chronic hepatitis B include cirrhosis and hepatocellular carcinoma, the latter being one of the most common cancers in the world.[7,8]

Since there is no effective treatment available for chronic hepatitis B, prevention is indispensable. Recombinant hepatitis B vaccines were the first recombinant vaccines approved for human use.[9] They were produced by subcloning the HBsAg gene into expression vectors that would direct the synthesis of large quantities of HBsAg in the host cells. The use of both yeast and mammalian host cells has been evaluated clinically. Approximately 20 to 25% of mammalian cell-derived HBsAg is glycosylated, while yeast-derived HBsAg is not.[10] Both preparations elicit protective antibodies comparably, and the yeast-derived vaccine has been preferred.[9] However, up to 10% of adults immunized against HBV still fail to respond to the currently used recombinant vaccines.[11]

HBsAg is also one of the most suitable markers to test the efficiency of an expression vector in mammalian cells. *In vivo*, HBsAg synthesis allows straightforward immunochemical detection of HBsAg gene expression in animals. HBsAg also elicits antibodies, permitting indirect demonstration of effective transduction by measuring antibody activity.

We examined the ability of the SV40-vector system to express a protein that would induce an immune response and as such, act as a vaccine. We report successful use of this gene delivery system to immunize mice against HBsAg. Significant levels of anti-HBs serum antibodies were found after the second inoculation. Importantly, we also found that the large T antigen (Tag)-deleted SV40-

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vector itself is not detectably immunogenic, even after eight inoculations.

Results

SV(HBS)

The principles used in the construction of Tag-deleted, replication-incompetent SV40 derivative viruses bearing transgenes such as hepatitis B surface antigen gene, SV(HBS), have been described.[2] Methods used to construct and propagate SV(HBS), as well as the specifics of the HBsAg gene included, are described in Materials and methods. A map of the SV(HBS) genome is shown in Figure 1.

Detection of HBsAg transcript in TC7 cells infected with SV(HBS)

TC7 cells were infected with SV(HBS) and control cells were mock-infected. Total RNA was extracted from infected cells, electrophoresed, transferred to a nylon membrane, and hybridized with a cDNA probe for the HBsAg gene. Expression of HBsAg was detected in SV(HBS)-infected cells but not in mock-infected cells (Figure 2). Two transcripts were observed: a 1 kb HBsAg transcript, plus a larger mRNA of 4.6 kb. The latter most likely represents read-through transcription, which we often observe in this system. As control, the same blot was stripped and hybridized with a cDNA probe for GAPDH, which showed expression from both treatment groups (Figure 2).

Expression of HBsAg was confirmed by RT-PCR analysis. Total RNA was extracted from SV(HBS)-infected and SV(luc)-infected TC7 cells and used as a template for RT-PCR. RT-PCR negative control was also performed without RNA template. The expected product of 438 bp was identified only from the cell sample infected with SV(HBS). It was not observed when RNA was pretreated with RNase before RT-PCR. RNA from TC7 cells infected with an unrelated SV40-derivative virus, SV(RGP) (see Materials and methods), was negative for HBsAg transcript (Figure 3).

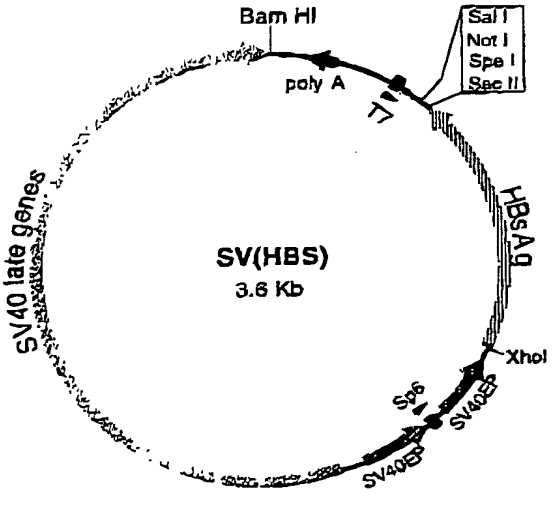

Figure 1 Map of SV(HBS). This recombinant viral genome is excised from pSV5(HBS) as a NotI fragment, and then recircularized (see Materials and methods). It contains two head-to-tail SV40 EP followed by HBsAg gene and the SV40 polyadenylation signal.

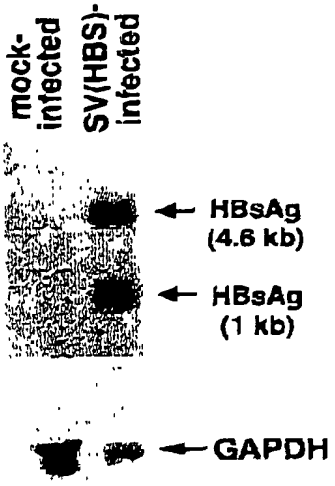

Figure 2 Expression of HBsAg gene in TC7 cells. TC7 cells were transduced with SV40 derivative virus containing HBsAg gene (SV(HBS)) at MOI of 10. Mock-infected cells were treated similarly in control preparation without the virus. RNA was harvested from these cultures, 24 h later, then electrophoresed, blotted and hybridized with HBsAg probe (see Materials and methods). The second HBsAg transcript represents a read-through mRNA, as we have seen in many cases in this system. Its size is indicated. The same filter was stripped and reprobed with a control cDNA probe (GAPDH).

Detection of HBsAg in TC7 cells in vitro

To determine whether detectable levels of HBsAg were produced in cultured cells on infection with SV(HBS), TC7 cells were infected at a MOI of 10. Control cells were mock-infected. SV(HBS)-infected and mock-infected cells were harvested 24 h later. Cell lysates were electrophoresed, blotted, and treated with anti-HBs rabbit polyclonal antiserum, followed by HRP-conjugated goat anti-rabbit antibody. HBsAg was detected by chemiluminescence in SV(HBS)-infected cells but not in mock-infected cells (Figure 4). HBsAg was also detected by immunostaining as described elsewhere.[5]

The sizes of the HBsAg protein bands produced by infecting with SV(HBS) were slightly different from the sizes of the bands detected within recombinant HBsAg. These differences may reflect different post-translational modifications (eg glycosylation). The presence of multiple bands may reflect the tendency of different preparations of HBsAg that were prepared differently to undergo variable oligomerization.

Detection of anti-HBs in mouse serum

To determine whether the SV40-derived virus could produce HBsAg *in vivo* and therefore elicit detectable anti-HBs in mice, BALB/cJ mice were treated in three groups of five mice each: intraperitoneal (i.p.) inoculation of 10^8

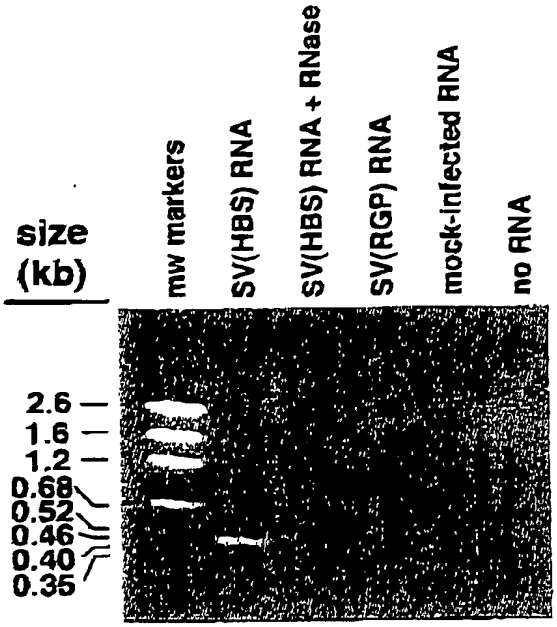

Figure 3 RT-PCR amplification of HBsAg transcript in TC7 cells. TC7 cells were transduced with SV(HBS) at a MOI of 10. Control cells were mock-infected or, were infected with a different SV40-derived virus (SV(RGP)), containing the gene for the major glycoprotein of rabies virus, but lacking HBsAg. RNA was harvested from these cultures 24 h after infection. An aliquot of RNA from SV(HBS)-infected cells was treated with RNase before RT-PCR. RT-PCR was performed using primers within the HBsAg gene (see Materials and methods) RT-PCR negative control was performed without the RNA template. Molecular size markers (pGEM markers; Promega) are shown on the left

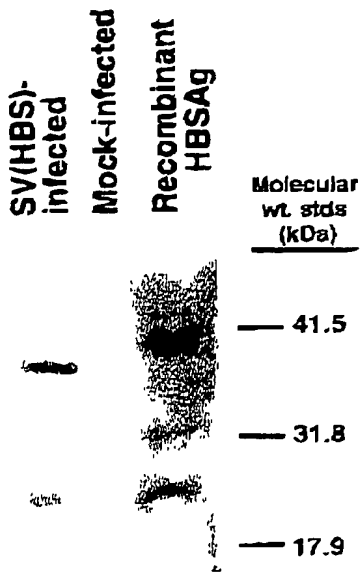

Figure 4 Expression of HBsAg in TC7 cells transduced with SV40 derivative virus containing HBsAg gene. SV(HBS) were used to transduce TC7 cells in vitro at a MOI of 10. Mock-infected cells were treated similarly in control preparation without the virus. Protein lysate was harvested from these cultures 24 h after infection (see Materials and methods). Sixty micrograms of cell lysate was electrophoresed by SDS-PAGE, blotted, then treated with anti-HBs rabbit polyclonal antibody followed by HRP conjugated goat anti-rabbit antibody. Signal was detected by chemiluminescence assay

IU per mouse, subcutaneous (s.c.) inoculation of 10^8 IU per mouse, and vehicle (DMEM/2% FBS) only. Mice were bled every 2 weeks to collect sera. Inoculation was done after bleeding, every fourth week. Each serum sample was tested for antibody to HBsAg by ELISA. Levels of serum anti-HBs were increased significantly following the second injection, and remained elevated in both i.p.- and s.c.-injected mice (Figure 5).

The antibody, so produced was also characterized by Western blot analysis using commercially supplied HBsAg (Scantibodies Laboratories, Santee, CA, USA) (Figure 6).

In these blots, the murine antisera, as well as the rabbit antiserum, all recognized the 24 kDa recombinant protein. Higher molecular size derivatives or oligomers present in this recombinant protein preparation were also recognized by the control rabbit antiserum but not by sera from the mice receiving SV(HBS).

Neutralizing antibody vs SV40 is not detectable

We tested the same sera to see whether SV40-derived virus itself was an effective antigen *in vivo*. SV(HBS) was incubated with the same murine sera that tested positively for anti-HBs, and then used to infect TC7 cells. The treated cells were tested for SV40 infection by *in situ* PCR. This assay, performed on microscope slides of virus-treated cells, allows direct visualization and both reproducible and rapid enumeration of SV(HBS)-infected cells. Sera from i.p.- and s.c.-inoculated mice did not neutralize SV40 infectivity differently from prebleed sera, even after eight inoculations (Figure 7). Control recipient sera, similarly, failed to neutralize SV40 infectivity. Simultaneously, rabbit antiserum to SV40 (diluted 1:400, see Materials and methods) neutralized >94% of virus infectivity, as our positive control. Pre- and post-inoculation sera from mice that received SVluc failed to neutralize SV(HBS) (not shown).

Discussion

Nonreplicating recombinant viruses have been generated for the purpose of vaccination, principally using poxviruses for veterinary applications. Most notably, vaccinia virus based-recombinant vaccines have been used against, for example, vesicular stomatitis, influenza, and rabies viruses.[12–16] Additional poxviral vectors have also been used for immunization, including fowlpox,[17–19] canarypox,[20] capripox[21] and pigeonpox.[22] In humans, canarypox-based vaccines expressing antigens from rabies virus[23] and HIV[24,25] have been shown to be safe and immunogenic and other canarypox virus-based vaccines are being assessed.

Several recombinant vaccinia viruses have been con-

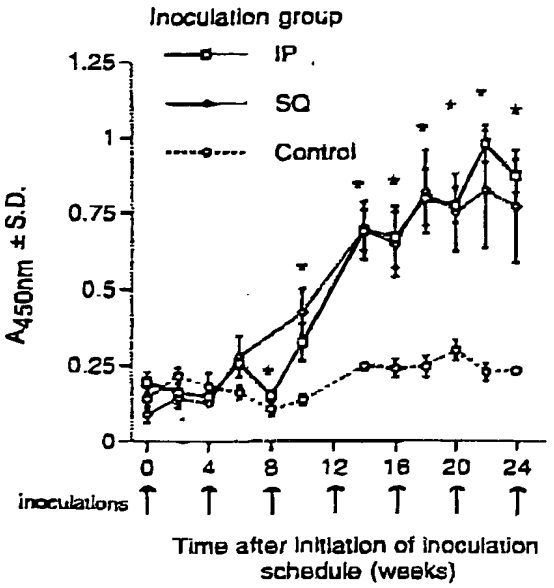

*Figure 5 Production of antibody against HBsAg in mice. SV(HBS) (10^8 IU) were used to inject mice every 4 weeks; five each of control, intraperitoneally and subcutaneously injected mice. These mice were bled every 2 weeks. Serum samples were diluted to 1:10 and antibody versus HBsAg were measured by ELISA assay (see Materials and methods). Individual serum samples were averaged for each treatment group and time point, ± s.e.m. IP; intraperitoneal injection, SQ; subcutaneous injection. *, $P < 0.05$, compared with control sera drawn at the same time point. Arrows indicate when SV(HBS) or control injection occurred after bleeding.*

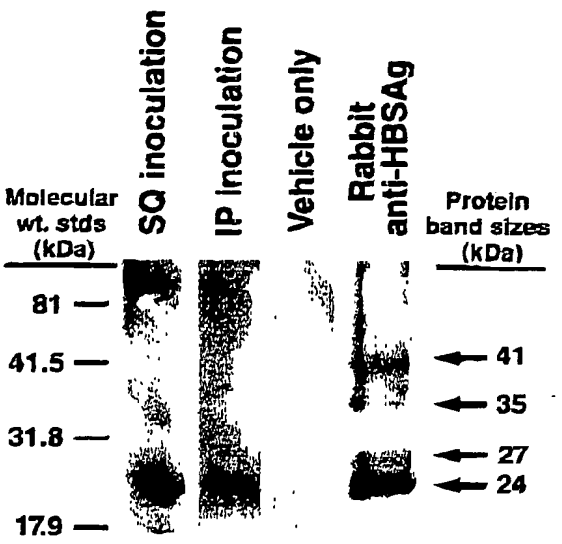

Figure 6 Characterization of antibody against HBsAg elicited by SV(HBS). Commercial HBsAg was electrophoresed on SDS-PAGE gel and blotted. Membranes were hybridized to serum from mice inoculated with SV(HBS), and then to HRP-conjugated sheep anti-mouse Ig. Signal was detected by chemiluminescence assay. IP; intraperitoneal injection, SQ; subcutaneous injection.

structed that direct the synthesis of HBsAg *in vivo*.[26,27] Chimpanzees infected with vaccinia virus-HBV recombinant were protected against HBV challenge.[28] Recombinant adenovirus also produced HBsAg *in vitro*[15] and elicited anti-HBs in rabbits[29] and hamsters.[30]

The HBV genome consists of a small (approximately 3.2 kb), partially double-stranded, circular DNA molecule.[31] HBsAg is made up of a group of viral structural proteins that constitute the principal components of subviral particles, filaments and the viral envelope.[25,31] The HBsAg gene is one of the four major open reading frames (ORF). It contains three in-frame translation initiation codons that divide the gene into three coding regions known as preS1, preS2 and S. The three polypeptides produced are known as the major (S), middle (preS2 + S), and large (preS1 + preS2 + S) proteins. The major S protein is the most abundant form, and exists as a glycoprotein of 27 kDa (GP27) and as a nonglycosylated 24 kDa protein (P24). PreS1(P39/GP41) proteins and preS2 (GP33/GP36) proteins constitute approximately 2% and 15% of the HBsAg proteins produced from this ORF.[10] Due to high cysteine content, these HBsAg proteins form mixed dimers or oligomers after dissociation with mercaptoethanol, which may yield complicated patterns and slower migration on SDS-PAGE.[26] Repeated freezing and thawing can also cause spontaneous aggregation. This tendency to mixed oligomerization may

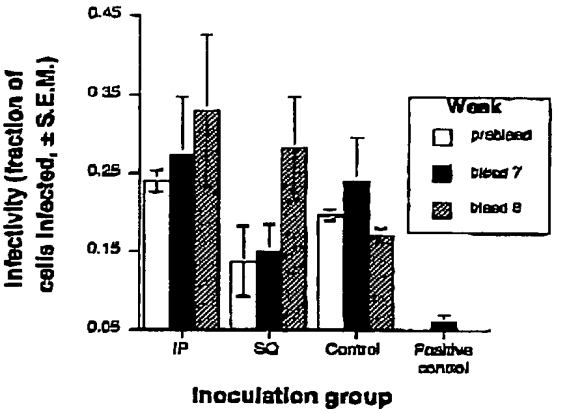

Figure 7 Production of antibody against SV40 in mice. SV(HBS) was incubated with mice sera diluted to 1:100, before infection to TC7 cells. The proportions of TC7 cells infected was determined by in situ PCR (see Materials and methods). IP; intraperitoneal injection, SQ; subcutaneous injection. Within each group, the fraction of infected cells was not significantly different in comparing preblead and post-inoculation samples. Rabbit anti-SV40 serum (kindly provided by Dr JS Butel) served as the positive control. This serum was tested at a 1:400 dilution, and the fraction of cells infected following treatment with this antiserum was significantly different ($P < 0.01$) from all other groups tested.

account for the diverse banding patterns noted in our Western blots, including slower migration seen in some of our studies.

Different methods of preparation may be reflected both by different banding patterns seen when the same sera were used to probe HBsAg produced by TC7 cells and HBsAg produced by recombinant methods in bacteria (eg Figure 5). The fact that HBsAg delivered to eukaryotic cells may differ somewhat in structure from HBsAg derived from bacteria may also account for the differences in banding patterns seen when recombinant HBsAg is probed by our mouse antisera, compared to commercial rabbit antisera.

SV40 is a small (5.2 kb) nonenveloped double-stranded DNA virus of the papilloma virus group. A number of features of SV40 are advantageous for use as a gene transfer vehicle: (1) It infects and express its genes in cells from variety of tissues and of different animals. (2) Its genome may persist in infected cells, either by integration or as a minichromosome.[4] (3) Live, wild-type SV40 was an unrecognized contaminant in early lots of polio vaccines, and was inadvertently administered to many polio vaccine recipients. However, extensive epidemiologic studies on polio vaccine recipients have not shown any significant adverse effect of live wild-type SV40 in humans.[32,33] (4) In addition, as shown in the present work, the administered SV40 virus particle itself elicits no detectable neutralizing antibody response by itself. Animals in which the virus replicates may produce antibodies against these proteins. In the absence of virus replication, we did not detect antibodies capable of neutralizing the virus' infectivity.

SV40 encodes five major proteins, including three structural genes and large and small T antigens.[34] The transforming property[35] and the principal target of immunity *vs* SV40-infected cells is the large T antigen.[36] T antigen is necessary for late virus gene expression and for virus DNA replication. However, we have found that T antigen-deficient SV40 can be grown easily to a relatively high titer (>10^9 infectious units per milliliter) in COS cells, which supply the missing T antigen *in trans*.[1,2] (5) The efficiency of transduction and level of expression are known to be very high. Thus, using HBsAg, we examined the use of this SV40 system for the introduction of genes *in vitro* and *in vivo*.[1]

The purpose of these studies was to test the ability of SV40 to deliver DNAs encoding important antigens, such as HBsAg. Our data show that SV(HBS) elicited high levels of antibody against HBsAg. Mice receiving a single inoculation did not induce detectable antibody within 4 weeks. While additional bleeds at later times may have allowed us to detect serologic immune responses, a second inoculation was performed 4 weeks after the first and it elicited detectable levels of antibody. Anti-HBs antibody activity in immunized mice remained above control and preblced levels thereafter. Antibody activity increased with additional inoculations. We did not test cell-mediated immunity.

Our data show no evidence of neutralizing antibody against SV40. We used BALB/c mice, which have been shown to produce high levels of antibody when inoculated with wt SV40.[37,38] Most antibody responsiveness against wt SV40, and the most studied antibody responses, are against Tag.[39,40] Antibody activity against the structural proteins of SV40 is most readily measured by virus neutralization assays, similar to those done here. In some cases, wt SV40 and related papovaviruses have been reported to elicit neutralizing antibodies, usually at low levels.[41] However, these responses are generally small in comparison to antibody responses to Tag[42] and the extent to which antibody responses *vs* virus structural proteins may require virus replication and/or Tag expression is not clear.

We found no evidence of such antibodies in our studies. SV40 is not phagocytosed like other large particulates. Rather, the virus interacts with a cell membrane receptor, thought to be class I MHC,[43,44] to yield an endocytic vesicle that goes directly to the nucleus, where the virus uncoats.[45] Antigen processing in phagolysosomes is not clearly documented for SV40. This observation, together with the lack of Tag and the consequent lack of late gene expression in infected cells, may account for the absence of detectable antibody responses to capsid antigens.

In considering the potential for Tag-deleted SV40 as a gene transfer vehicle, the most important operational issues to be resolved regarding immunogenicity center on the antigenicity of infected cells and the antigenicity of the inoculated virus particle. In the one case, immunogenicity of infected cells will limit the longevity of transgene expression in immunocompetent hosts. In the other, immune responses against the virus particle may restrict the potential for multiple inoculations. Our studies to date suggest that Tag-deficient SV40 may be useful as a gene transfer vehicle on both accounts.

Materials and methods

Cell lines

Two monkey cell lines were used: COS-7 cells (ATCC, Bethesda, MD, USA) which contain an origin-defective SV40 genome, and TC7 cells which do not. Both cell lines were maintained by serial passage in Dulbecco's modified Eagle's medium (DMEM)-10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA).

Mice

Retired female bleeders, BALB/cJ (Jackson Laboratories, Bar Harbor, ME, USA), were fed and housed in accordance with American Association for Accreditation of Laboratory Animal Care standards.

Generation of recombinant SV40

The HBsAg gene was cloned into pSV5, which yielded pSV5(HBS). The details of the construction of this plasmid is described elsewhere.[3] Briefly, pSV5 consists of the SV40 genome, minus the T antigen gene, plus a polylinker immediately downstream of two tandem SV40 early promoters (EP). This viral genome was cloned, using an engineered *Not*I site in the polylinker, into pGEM13. A 739 bp DNA fragment containing the coding sequences of the HBsAg major protein gene (see Discussion) was amplified by polymerase chain reaction (PCR) using oligonucleotide primers that contained orienting restriction sites; HBVS1 (5'-TTCTCGAGGAT TGGGGACCC-3') and HBVS2 (5'-CGACCGCGGCTC TTTCTTTTGTTA-3'). This HBsAg DNA encoded a 24 kDa protein of 226 amino acids.

The PCR product was digested with *Xho*I and *Sac*II and

subcloned directionally to the pSV5 polylinker. Recombinant SV40 derivative virus, SV(HBS), was produced by excising the recombinant virus genome from pSV5(HBS) with *NotI*, band-purifying it, then recircularizing the modified viral genome, and transfecting it into packaging cell line (COS-7 cells) (Figure 1). The anticipated transcript length for this transgene is 1 kb, including the untranslated 3' end of the HBsAg transcript which was joined to 0.2 kb of untranslated SV40 sequences before the polyadenylation signal. The entire SV(HBS) virus genome is 3.6 kb.

Two weeks later, crude virus was obtained by three rounds of freezing–thawing the cells, followed by 2.5 min sonication. This lysate was used to produce working stocks of virus by infecting more COS-7 cells.

Infection, 2 h at room temperature, was done in a small volume of culture medium (DMEM/2% FBS). After infection, more medium was added and cultures were incubated for 2 weeks at 37°C, 7% CO_2 until approximately 50% of cells were infected as judged by cytopathic effect. Virus was harvested by freezing–thawing and sonication, as described above.

SV(RGP) was used as a control virus in some of these studies. It lacks HBsAg. Instead, SV(RGP) contains the gene for the major glycoprotein antigen of rabies virus (a gift from Dr H Koprowski, Thomas Jefferson University, PA, USA), cloned downstream from the cytomegalovirus intermediate-early promoter. Production and expansion of SV(RGP) stocks was carried out similarly to that for SV(HBS). SVluc, an additional control virus used in some of our studies, contains the firefly luciferase transgene instead of HBsAg. Its production and characteristics have been published.[25]

Measuring infectivity of SV(HBS) by in situ PCR

Virus was titrated using TC7 cells, by *in situ* PCR, an approach we devised specifically to measure the infectivity of replication-incompetent DNA viruses.[46] Appropriate dilutions of SV(HBS) in DMEM/2% FBS were used to infect TC7 cells in 2.5-cm wells. Each well was layered with 100 µl of diluted virus stock and 400 µl of DMEM/2% FBS, and was incubated for 2 h at room temperature, shaking. More medium was added and cultures were incubated for 24 h more. Cells were trypsinized and placed on 3-amino-propyl-triethoxy-silane treated Teflon slides (Erie Scientific, Erie, PA, USA). Slides were fixed with 4% paraformaldehyde, then treated with proteinase K (6 µg/ml). Each slide was covered with PCR cocktail (250 mM each of dNTP, 1 µM each of primer, 0.1 U/µl of *Taq* polymerase, 500 mM KCl, 100 mM Tris pH 8.3, 15 mM $MgCl_2$ and 0.01% gelatin). The primers were BP3–1 (5'-ACTGTCACTGGTGTGAGCCCTG-3') and BP3–2 (5'-TCGACCCCAATGTCTGGGGTC-3'). The slide was covered with cover slips, sealed with nail polish, and incubated as follows: 94°C for 1 min, 50°C for 1.5 min, and 72°C for 1 min for 35 cycles (PT-100; MJ Research, Watertown, MA, USA). After removing the coverslip, the slide was hybridized (50% formamide, 2 × SSC, 10 × Denhardt's solution, 1 mg/ml salmon sperm DNA, 0.1% SDS, 32 mM 5' biotinylated probe) overnight at 37°C. The color of the slide was developed by incubation at 37°C with 330 mg/ml streptavidin-peroxidase and then with a reaction solution (50 mM sodium acetate pH 5.0, 0.15% H_2O_2 and 5% 3-amino-9-ethyl-carbazole (Sigma, St Louis, MO, USA).

Detection of HBsAg transcript by Northern analysis

TC7 cells were infected with SV(HBS) at MOI of 10. Twenty four hours later, total RNA was prepared from cultured cells (RNAzolB; Molecular Research Center, Cincinnati, OH, USA), and electrophoresed (10 µg per lane) in formaldehyde gels, blotted to nylon membrane (Amersham, Arlington Heights, IL, USA). *XhoI* to *SacII* HBsAg fragment was labeled with α^{32}P-dCTP (DuPont NEN) for DNA probe. The membrane was baked for 2 h at 80°C, prehybridized and hybridized at 42°C (solutions include 50% formamide, 0.1% SDS), and washed stringently (final wash: 0.1 × SSC, 0.1% SDS, 50°C). Binding of radiolabeled probes was visualized by autoradiography.[47]

Detection of HBsAg transcript by RT-PCR

Total RNA from cultured cells was obtained as above. PCR was done in 50 µl containing 1 µg of total RNA, 1 µM each of primers HBVS3 (5'-CTAGACTCGTCC TGGACTTC-3') and HBVS4 (5'-CACTAGTAAACT CAGCCAGG-3'), 0.1 U/µl each of AMV reverse transcriptase and Tfl DNA polymerase (Promega, Madison, WI, USA), 0.2 mM each of dNTP, 1 mM $MgSO_4$ and AMV/Tfl reaction buffer (Promega, Madison, WI, USA). It was incubated as follows: 45 min at 48°C, 2 min at 94°C, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, and 68°C for 1 min.

Detection of HBsAg protein by Western blot analysis

Cells were lysed with lysis buffer (100 mM Tris pH 8.0, 100 mM NaCl, 0.5% NP-40, 1 mM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 10 µg/ml pepstatin). Protein lysate was loaded on a 12% SDS-polyacrylamide gel, electrophoresed and blotted to a PVDF membrane (Schleicher and Schuell, Keene, NH, USA). The membrane was blocked with 5% skim milk for 2 h, treated with rabbit polyclonal anti-HBs (1:1000 in 1% BSA/PBST; Accurate Chemical and Scientific, Westbury, NY, USA) overnight at 4°C, and then with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:1000 dilution with 1% BSA/PBST; Transduction Laboratories, Lexington, KY, USA) for 2 h at room temperature. Signal was detected with chemiluminescence reagent (DuPont NEN, Wilmington, DE, USA).

Detection of anti-HBs by enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well plates were coated with 1 µg per well synthetic peptides (surface antigen peptide 28)[48] in PBS/10% FBS. Fifty microliters of diluted serum samples (1:10 in PBS/10% FBS) were transferred into corresponding wells for overnight incubation at 4°C. Each well was washed six times with PBS, treated with HRP-conjugated goat anti-mouse Ig (1:1000 dilution). The plates were incubated for 1 h at 37°C, washed six times in PBS and then substrate (1.5 mg/ml *o*-phenylenediamine dihydrochloride in 0.05 M phosphate-citrate/0.014% H_2O_2) added. Spectrophotometric readings at 450 nm were taken after 15 min of color development (Dynatech MR5000 ELISA plate reader, Alexandria, VA, USA).

Detection of anti-HBs by Western blot analysis

The above protocol for Western blotting was applied. Purified HBsAg protein (0.4 µg) (Scantibodies Laboratories) per lane was electrophoresed (SDS-12%

PAGE) and blotted to PVDF membrane (Schleicher and Schuell), treated with test mouse serum (1:50 in 1% BSA/PBST) and then to HRP-conjugated sheep anti-mouse antibody (1:5000 in 1% BSA/PBST; Amersham). Signal was detected with chemiluminescence reagent (DuPont NEN).

Testing for neutralizing activity versus recombinant SV40 by in situ PCR

One microliter of SV(HBS) (10^9 IU/ml) was incubated with 99 µl of each mouse serum (1:100 dilution in DMEM/2% FBS) for 2 h at 37°C. This was added to TC7 cells in a 60 mm dish containing 400 µl of DMEM/2% FBS for *in situ* PCR (described above) to deduce the proportion of infected TC7 cells.

Anti-SV40 serum

Rabbit anti-SV40 serum was the kind gift of Dr J Butel (Baylor College of Medicine). It is capable of neutralizing >50% of wt SV40 plaque forming activity at dilutions >1:400, the dilution used in our virus neutralization assay.

Acknowledgements

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1364 **GENE TRANSFER TO THE LIVER IN VIVO USING REPLICATION-DEFICIENT RECOMBINANT SV40 VECTORS RESULTS IN LONG-TERM AMELIORATION OF JAUNDICE IN GUNN RATS.** B Parashar[1], BV Sauter[1], N Roy Chowdhury[1], A Kadakol[1], J Milano[2], D Strayer[2] and J Roy Chowdhury[1]. [1]Departments of Medicine and Molecular Genetics, and the Marion Bessin Liver Research Center, Albert Einstein College of Medicine, New York, and [2]Department of Pathology, Jefferson Medical College, Philadelphia, PA.

A desirable vector for liver-directed gene therapy should transfer genes into non-dividing hepatocytes in vivo efficiently and should either integrate into the host genome, or be non-immunogenic so that it can be readministered. Current gene transfer vectors lack one or more of these characteristics. To generate a non-replicative, non-immunogenic vector, we have replaced the large T antigen (Tag)-encoding region of a plasmid containing the simian virus 40 (SV40) genome with the coding region of human bilirubin-UDP-glucuronosyltransferase (bilirubin-UGT). The plasmid was transfected into a monkey kidney cell line expressing Tag (COS-7) to generate a helper-free recombinant SV40, SV-hBUGT. Infectious titers of the virus, determined by in situ RT-PCR after infecting a non-Tag-expressing monkey cell line (TC-7), was 1-5 x 10^{10} per ml. A recombinant SV40 expressing firefly luciferase (SV-luc) was used as control. The vectors ($5x10^{10}$ infectious units) were infused into portal vein of bilirubin-UGT-deficient jaundiced Gunn rats through an indwelling catheter. After 10-30 days, liver biopsy specimens from the SV-hBUGT-treated Gunn rats, but not from controls, were positive for the transgene (by DNA PCR), human bilirubin-UGT mRNA (by RT-PCR) and bilirubin-UGT protein (by Western blot using a human bilirubin-UGT-specific monoclonal antibody). HPLC of bile showed excretion of bilirubin glucuronides (mainly monoglucuronide) in treated rats, demonstrating in vivo bilirubin-UGT activity. Mean serum bilirubin concentrations declined by 40% in the treated group in 2 weeks after the infusion and remained at that level throughout the study period (50 days). There was no evidence of antibody response against SV40 proteins or lymphocytic infiltration of the liver. Gene transfer experiments in cultured cells suggested random integration of the vector into the host genome. **CONCLUSION:** The recombinant SV40 is a non-replicative, non-immunogenic vector, that transfers genes efficiently into quiescent hepatocytes in vivo, and may represent a significant advance over current gene therapy vectors.

RECOMBINANT SIMIAN VIRUS 40 VECTORS INTEGRATE INTO HOST GENOME, AND PERMIT EFFICIENT, LONG-TERM AND REPEATABLE GENE TRANSFER TO THE LIVER *IN VIVO* B Parashar, S Ghosh, A Kadakol, B V Sauter, M Takahashi, N Roy Chowdhury, Jayanta Roy Chowdhury, Albert Einstein Coll of Medicine, Bronx, NY; J Milano, D Strayer, Jefferson Med Coll, Dept of Pathology, Philadelphia, PA

We have shown previously that the recombinant simian virus 40 (rSV40), in which the coding regions for the T antigens is replaced by a transgene, can transfer genes to the liver *in vivo*. The present study was aimed at determining (a) whether rSV40 is immunogenic, (b) if rSV40-mediated gene transfer can be repeated and (c) whether the transgene integrates into the host genome. One, 3 or 7 doses of SV-hBUGT ($3.6 \cdot 10^{10}$ IU) were infused into the portal vein of Gunn rats through indwelling catheters, resulting in 40%, 65% and 70% reduction in serum bilirubin levels, respectively, which persisted throughout the period of observation (2 months). The rat sera were negative for antibodies against purified SV-hBUGT, as determined by ELISA and immunoblot. There was no increase in serum alanine aminotransferase levels in the recipient rats and no lymphocytic infiltration was found in liver biopsy specimens, despite repeated injections of rSV40. To determine if the gene transfer can be repeated, an rSV40 expressing HBsAg was infused into the portal vein of naive rats or rats that had received 3 infusions of SV-hBUGT two weeks earlier. Immunoblot of liver homogenates showed equal level of HBsAg expression, demonstrating repeatability of gene transfer. Southern blot analysis of DNA extracted from livers of Gunn rats that had been treated with SV-BUGT 3-5 months earlier showed that the transgene (hBUGT) migrated with high molecular weight genomic DNA. The restriction analysis pattern indicated random integration of the transgene, and suggested that the recombinant viral genome is integrated as single copies, rather than as tandem inserts. **Conclusions:** Recombinant SV40 transfers transgenes efficiently into quiescent hepatocytes *in vivo*. The virus appears to integrate randomly into the host genome and is non-immunogenic, permitting long-term and repeatable gene transfer. Supported partly by NIH grants: DK 46057 (to JRC), DK 34357 (to NRC), AI41399 and RR13156 (to DSS) and Liver Research Core Center (P30-DK 41296).

Retrovirally Transduced Antisense Sequences Stably Suppress P210$^{BCR-ABL}$ Expression and Inhibit the Proliferation of BCR/ABL-Containing Cell Lines

By P. Martiat, P. Lewalle, A.S. Taj, M. Philippe, Y. Larondelle, J.L. Vaerman, C. Wildmann, J.M. Goldman, and J.L. Michaux

There is now strong evidence that the BCR-ABL gene product of the Philadelphia chromosome (P210) plays a crucial role in the pathogenesis of chronic myeloid leukemia (CML). We have previously shown that introduction of antisense oligonucleotides into K562 cells could transiently block the expression of P210 and specifically inhibit cellular growth in culture. In this report, we describe the use of a retroviral vector to introduce selected antisense and sense sequences, first into murine B10 cells, previously rendered interleukin-3 (IL-3) independent by transfection of BCR-ABL sequences, and second into K562 cells. The antisense transcripts generated under the control of MoMLV promoter specifically killed B10 cells in the absence of IL-3 and inhibited P210 expression almost completely. In K562 cells, the antisense sequences led to a dramatic reduction of P210 expression and increased their doubling time by more than twofold. This effect was not reversed by the addition of exogenous IL-3 to the culture medium. Control HeLa or HL60 cells infected with the same constructs did not show any change in proliferation rate, despite abrogation of the normal BCR gene products. Rather unexpectedly, P210 suppression was not lethal in K562 cells, showing that such a cell line does not rely entirely on the expression of P210 for surviving, but depends on it as far as growth properties are concerned. We conclude that this approach can successfully achieve stable suppression of the oncogenic protein P210 and may be used to study further the mechanisms by which P210 is transforming cells. The effect on fresh CML cells in bone marrow cultures remains to be assessed before we can tell whether this technique may be used for selective suppression of leukemic hematopoiesis in vitro.

THE PHILADELPHIA chromosome, an acquired abnormality found in most chronic myeloid leukemias (CMLs), results in the formation of a hybrid BCR-ABL gene.[1-5] This gene is transcribed into an 8.5-kb hybrid messenger RNA (mRNA) that in turn is translated into a 210-Kd protein, P210, with deregulated tyrosine kinase activity.[6-8] The consistency of this finding and the fact that introducing this gene into murine hematopoietic cells can cause a CML-like disease[9-11] are strong evidence that P210 plays a crucial role in the pathogenesis of CML. Given this fact, one may speculate that inhibition of P210 expression could affect the leukemic phenotype of CML cells and could perhaps lead to new therapeutic approaches in this disease, at least in vitro. We have previously shown the effectiveness of antisense oligonucleotides directed against the 5' portion of the BCR-ABL mRNA in inhibiting P210 expression.[12] This inhibition resulted in growth suppression of the leukemic cell line K562. However, this inhibition was short-lived and the procedure required several consecutive electroporations. Other investigators have demonstrated the requirement for a functional BCR-ABL gene in the maintenance of the leukemic phenotype, using oligonucleotides targeted to the junctional sequences.[13] These experiments have shown the feasability of the antisense approach in achieving an antileukemic effect, but a stabler and long-lasting inhibition of P210 production may be useful for purging purposes or for studying the impact of P210 suppression on the phenotype of the leukemic cells. Therefore, we chose, as a next step, to use a retroviral-mediated antisense approach.

MATERIALS AND METHODS

Constructs

Vectors generating RNA molecules containing sequences complementary to the 5' portion of the hybrid BCR-ABL gene were constructed using standard recombinant DNA techniques.[14] A 585-bp *Sfi* I/*Bam*HI (Fig 1) restriction fragment (INS1) containing the ATG initiation codon was isolated from a plasmid containing the entire BCR-ABL sequences (KW3; provided by Dr G. Grosveld, Rotterdam, The Netherlands) and cloned both in a sense (pZ-S1) and antisense (pZ-AS1) orientation in the retroviral expression vector pZIP/Neo-SV(X).[15] The structure of the recombinants was verified by restriction enzyme digestion of isolated plasmid DNA. To generate cell lines producing the recombinant retroviruses, the amphotropic packaging cell line GP12+*env*AM-12[16] was transfected with either the sense or antisense constructs using the calcium phosphate transfection method.[14] Individual colonies were isolated in G418 (1.2 mg/mL), expanded, and assayed for the production of recombinant viruses by infection of NIH-3T3 cells. Production of recombinant RNAs was assayed in infected NIH-3T3 cells, after G418 selection, using Northern blot techniques[14] and the (^{32}P)-dCTP-labeled[17] insert as a probe. The second sense and antisense constructs (pZ/S2 and pZ/AS2), the effects of which are briefly described in the Results section, were made in the same way. The insert INS2 was a 200-bp junctional fragment, produced by PCR, cloned into the same vector and spanning exactly 100 bp on each side of the b3a2 junction (Fig 1) of the BCR-ABL cDNA.

Cellular Experiments

Because we expected P210 inhibition to cause the death of CML cells having integrated the antisense sequences, we chose, as a first target, the B10 lymphoid cell line, which has been rendered interleukin-3 (IL-3) independent by infection with a retroviral vector encoding P210 (b3a2-type junction) sequences.[18] In the event of inhibition of P210 expression, the cells could be rescued by addition of exogenous IL-3, thereby allowing to assay P210 expression on living material. As this cell line was shown to be very resistant to infection with the

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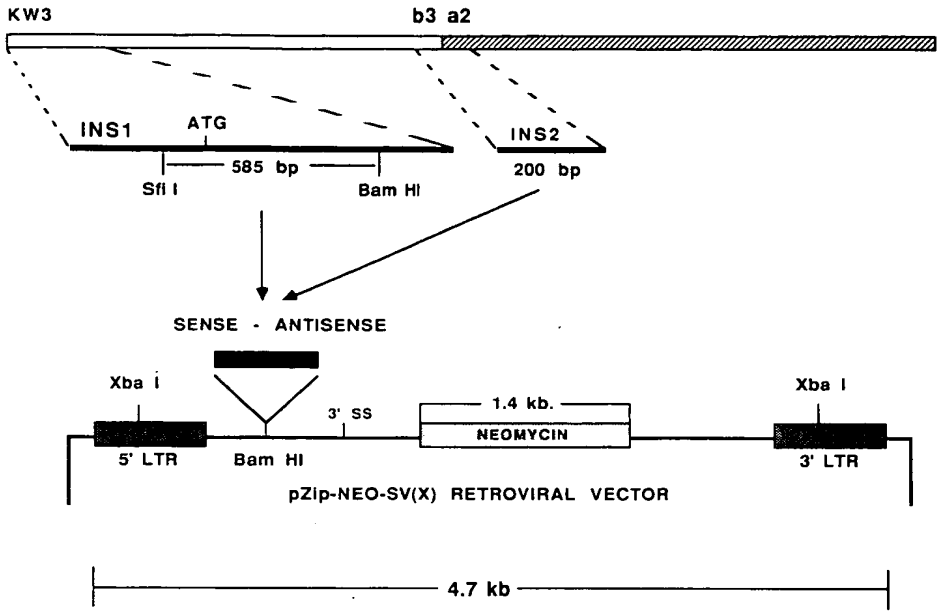

Fig 1. Retroviral constructs. Schematic description of the four retroviral constructs described in the text. The constructs pZip/S1 (sense orientation) and pZip/AS1 (antisense orientation) are made by introducing the 585-bp *Sfi* I/*Bam*HI restriction fragment of the BCR-ABL cDNA (INS1) into the *Bam*HI cloning site of the retroviral vector pZip/Neo-SV(X). The pZip/S2 and pZip/AS2 constructs derive from the introduction into the same cloning site of a 200-bp fragment (INS2) obtained by PCR and overlapping the b3a2 junction (100 bp on each side of the junction).

amphotropic constructs (data not shown) and as it already contained a neomycin-resistance gene[18] that did not allow for selection of cells infected with our constructs, two types of experiments were designed to assess the efficiency of the constructs: transient expression after electroporation for a rapid first screening of the constructs and stable expression, after cotransfection with a plasmid encoding hygromycin B resistance. The aim of the first experiments was to verify whether, during transient expression, there was a difference in the growth pattern of cells transfected with the sense and antisense constructs, before moving to the next step consisting of making stable transfectants. This first step involved only cell viability studies, at short term (4 days) and a P210 kinase assay. Constructs that seemed to have an effect (in this case, pZip/AS1) were characterized more precisely in the stable expression assay.

Transient Expression in B10 Cells

Transfection. The B10 cells were electroporated BioRad (UK) Gene Pulser, 960 mF, 250 V in 800 μL RPMI with 20 μg of pZip/Neo-SV(X), pZ/S1, or pZ/AS1. After electroporation, the cells were divided into two and grown in IL-3–supplemented and -deprived culture medium. Viable cells were counted at 24, 48, 72, and 96 hours after electroporation. WEHI-3B cells supernatant was used as a source of IL-3.

P210 assay. P210 assay was performed as described previously.[19] Briefly, the cells were washed in phosphate-buffered saline (PBS) and lysed in kinase lysis buffer. The lysates were cleared of insolvable material and immunoprecipitated with the anti-BCR monoclonal antibody, bcr (Ab-2), clone 7C6 (Oncogene Sciences). The immunoprecipitated proteins were labeled in vitro with (^{32}P)-γATP. The proteins were resolved on a 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was then dried and autoradiographed. The assay was performed on 4×10^6 living cells (Trypan Blue exclusion method), grown with IL-3 to ensure survival of cells in which P210 inhibition could occur, and collected 72, 96, and 108 hours after electroporation. The same type of experiments were performed using pZ/S2 and pZ/AS2.

Stable Expression in B10 Cells

Stable transfectants. Because the B10 cells are already carrying a neomycin resistance gene,[18] no further selection for the expression of the sense or antisense sequences was possible using the neomycin-resistance gene encoded by pZip/S1 or pZip/AS1. The B10 cells were

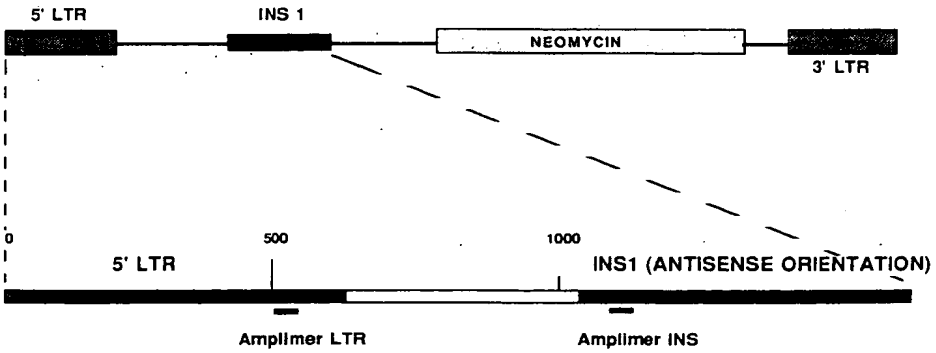

Fig 2. Schematic location of the oligoprimers used for the detection of the antisense message (AS1). Two primers were used, complementary to the antisense RNA (INS) and to the end of the LTR sequences (LTR). The reverse transcription procedure was primed using the INS oligonucleotide.

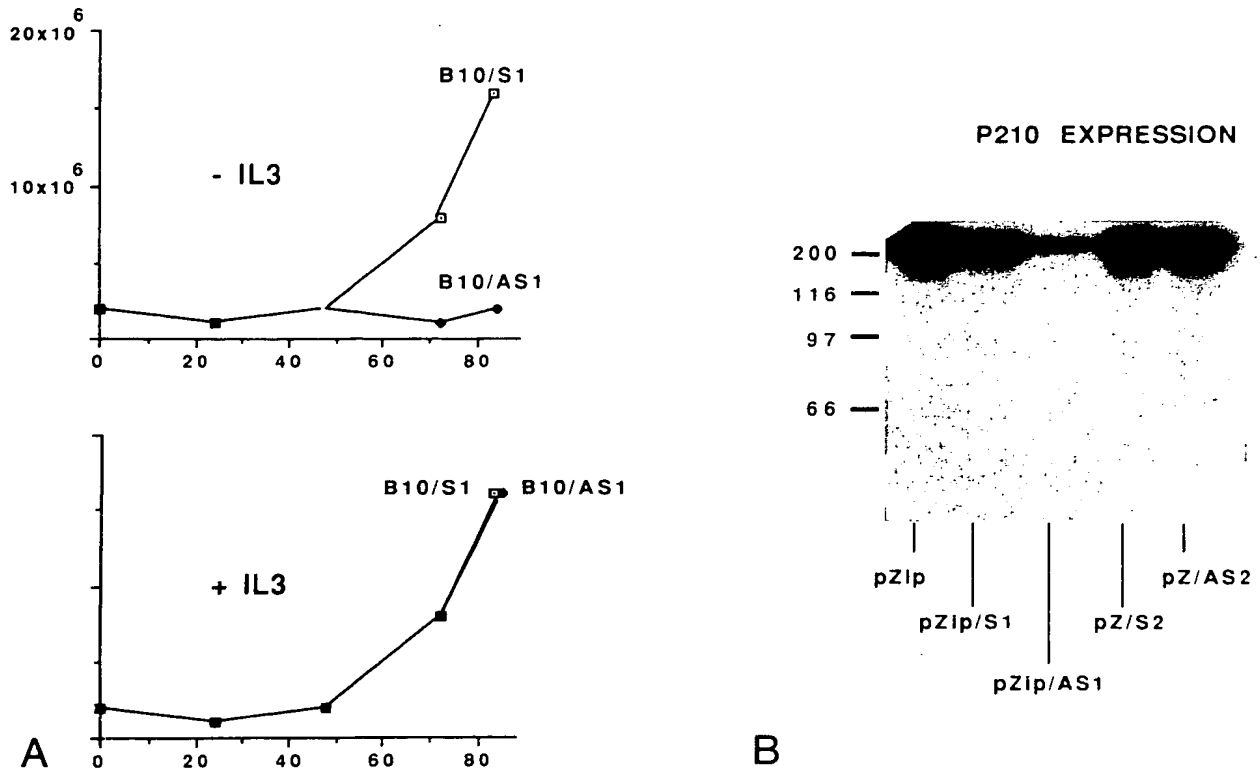

Fig 3. Transient expression of the constructs in B10 cells. (A) Proliferation of the B10 cells transfected with the sense (S1) or antisense (AS1) constructs in the absence (above) or presence of IL-3. The indicated numbers are the total numbers of living cells, as assessed by Trypan Blue exclusion. Time is in hours, starting from electroporation. (B) Comparison of the amount of P210 in 4 × 10⁶ living cells collected 72 hours after electroporation. The lanes pZ/S2 and pZ/AS2 show the results obtained with the second antisense construct (INS2) that was not efficient in inhibiting P210 expression.

therefore coelectroporated, as described above, with two plasmids, pZ/AS1 or pZip/S1, and a plasmid carrying the sequences encoding hygromycin B resistance. After 2 days, the cells were selected using hygromycin B (200 μg/mL). Resistant cells were then cloned, using the limiting dilution methodology. Individual clones were screened for the presence of the constructs S1 and AS1 using polymerase chain reaction (PCR) performed on DNA. The whole procedure was performed in IL-3–supplemented medium.

Proliferation studies. Several culture experiments were performed on the selected sense or antisense clones, in the presence or absence of IL-3. After growth in IL-3–supplemented medium, B10/S1 and B10/AS1 were washed with PBS and put back into culture medium with or without IL-3. The cells were then counted every day. Cell death was assessed using Trypan Blue exclusion. This procedure was repeated at least three times on every selected clone.

P210 measurements. These experiments were repeatedly performed on 4 × 10^6 living cells (B10/S1, B10/AS1, and unmanipulated B10 cells) using the procedure described above. HeLa cells extracts were used as a negative control to evaluate the specificity of the assay.

Southern blot studies. DNA was extracted from B10/S1 and B10/AS1 cells using standard procedure,[14] digested with *Xba* I, which cuts within the 5′ and 3′ long terminal repeat (LTR) of the constructs encoding the BCR-ABL sequences[18] and the sense/antisense constructs used in the transfection procedure. The digestion products were electropheresed through a 0.8% agarose gel, transfered to nylon membrane, and hybridized to the (^{32}P)-dCTP–labeled[17] 5′ insert (INS1) as a probe. This probe will pick up both the BCR-ABL sequences and the sense/antisense sequences. This, together with the PCR studies described below, were performed to ensure, as much as we could, that clones having lost the expression of the BCR-ABL mRNA after a genomic rearrangement had not been selected by chance during the cloning procedure.

PCR studies. Clones of B10 cells stably transfected with either the sense (B10/S1) or the antisense (B10/AS1) construct were investigated for expression of BCR-ABL mRNA and antisense sequences as follows. Total RNA was extracted from B10/AS1 and B10/S1 cells according to the method described by Chomczynski and Sacchi.[20] RNA was first subjected to DNAse I treatment (to avoid amplification of the integrated BCR-ABL or antisense DNA), re-extracted, and reverse transcribed using either a primer complementary to the antisense RNA:INS (Fig 2) or to the BCR-ABL exon a3 RNA:A3. As a control, the same procedure was performed without adding reverse transcriptase. The samples were amplified using a couple of primers spanning the BCR-ABL junction (B1/A3) during 25, 30, and 35 cycles of amplification, as previously described,[21] or a couple of amplimers (LTR/INS) located on the antisense construct (Fig 2) during 30 cycles of amplification. The 3′ primer, INS, was complementary to the antisense RNA only. If a BCR-ABL message is present, the B1/A3 primers pair will produce a 395-bp fragment for a b3a2 junction and the LTR/INS pair will amplify a 551-bp fragment for a RNA in the

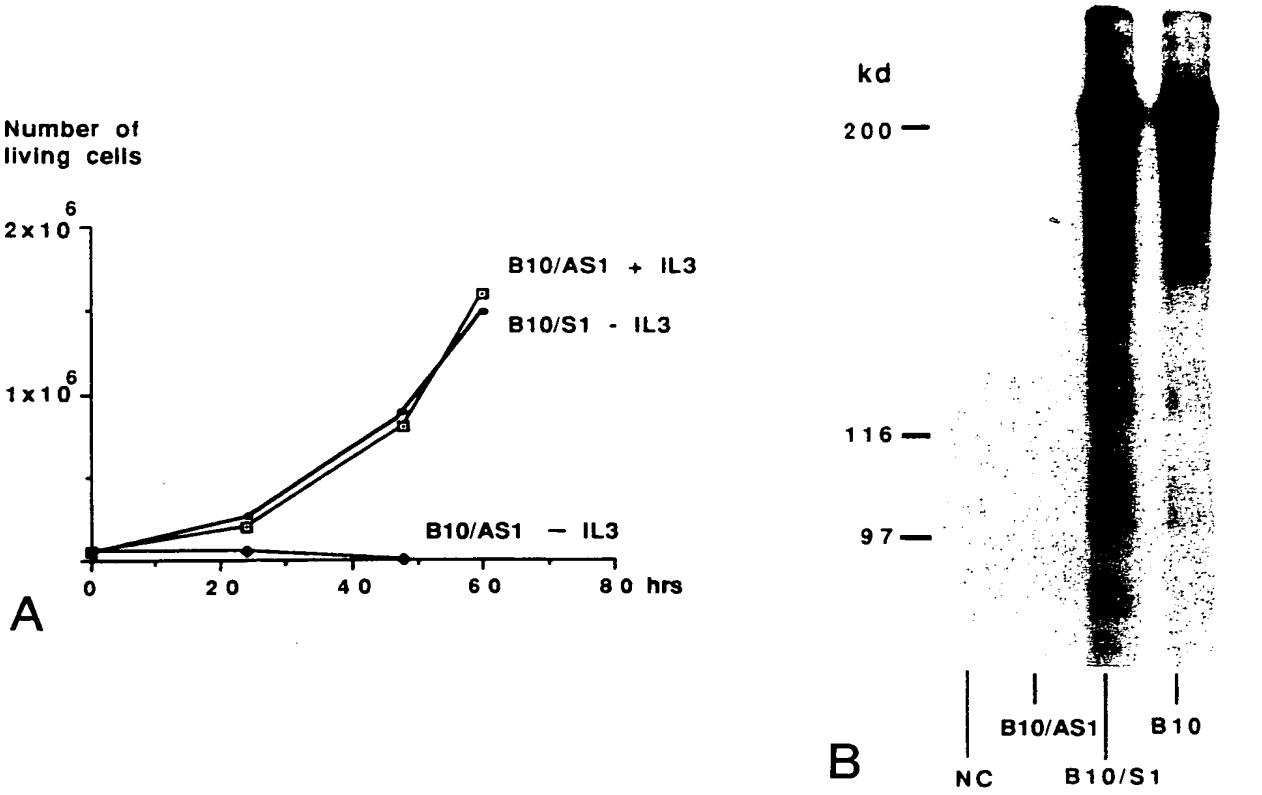

Fig 4. Stable expression in B10 cells: proliferation studies and P210 assay. (A) Comparison of the proliferation rate of B10 cells stably expressing the antisense sequences (AS1) in the presence (+IL-3) or absence (−IL-3) of IL-3. The proliferation rate of B10 cells expressing the sense sequences (−IL-3) is given for comparison. (B) P210 kinase assay comparing the protein expression in 4 × 10^6 B10 cells stably transfected with the antisense construct to its expression in the same number of unmanipulated B10 cells (B10) and in the cells expressing the sense construct (B10/S1). The negative control consists of HeLa cells.

antisense orientation (AS1). The sequences of the amplimer sequences are as follows: A3, 5′-GTGATTATAGCCTAAGACCCGGAGC-3′; B1, 5′-GAAGAAGTGTTTCAGAAGCTTCCTCC-3′; LTR, 5′-TGTGGTCTCGCTGTTCCT-3′; INS, 5′-GAACGGGAC-GACCGGGGA-3′.

Northern blot studies. Total RNA was extracted according to the same protocol as described for RT-PCR studies. Twenty micrograms of total RNA was loaded onto a denaturing gel (agarose 0.7%, formaldehyde 3%), electrophoresed for 20 hours at 40 V in a MOPPS buffer, transferred to a nylon membrane using 10× SSPE, and hybridized with the (^{32}P)-dCTP–labeled[17] junctional insert (INS2) as a probe to pick up only BCR-ABL mRNA. The hybridization conditions were as follows: 48°C in 50% formamide with 5× SSPE.

Stable Expression in K562 Cells

Infection procedure. We next introduced the constructs pZ/S1 and pZ/AS1, the efficacy of which had been verified in the B10 stable expression assay, into K562 cells by retroviral infection followed by neomycin-resistance selection. K562 cells were cultured in the presence of the retroviral supernatants for 2 days. Four days after the beginning of the procedure, G418 (2.5 mg/mL) was added to the culture medium and resistant cells were selected. Cell growth rate measurements and P210 assays were performed on control K562 and K562 expressing the sense (K562/S1) or antisense sequences (K562/AS1). As a control for the specificity of any effect on growth properties, we used the same procedure to generate clones of HeLa and HL60 cells expressing the sense or antisense constructs.

Western blot studies. Experiments were performed on 4 × 10^6 unmanipulated K562, K562/S1, K562/AS1, or on the same number of HeLa/S1 and HeLa/AS1 using the monoclonal antibody bcr (Ab-2; Oncogene Sciences) according to the manufacturer's protocol, except that proteins were first immunoprecipitated with the Ab-2 monoclonal antibody, as in the kinase assay described above, before being loaded onto the polyacrylamide gel. This achieved better results in terms of BCR-related protein resolution.

RESULTS

Constructs

The titers of retroviral supernatants generated by the two chosen (GP12/pZ-AS1 and GP12/pZ-S1) clones, determined by resistance of NIH/3T3 fibroblasts to G418, were 4 × 10^4 and 6 × 10^4 colony-forming units (CFU)/mL, respectively. Northern blot studies, performed after G418 selection, on infected NIH/3T3 fibroblasts showed these cells to express a high level of the recombinant RNAs (data not shown).

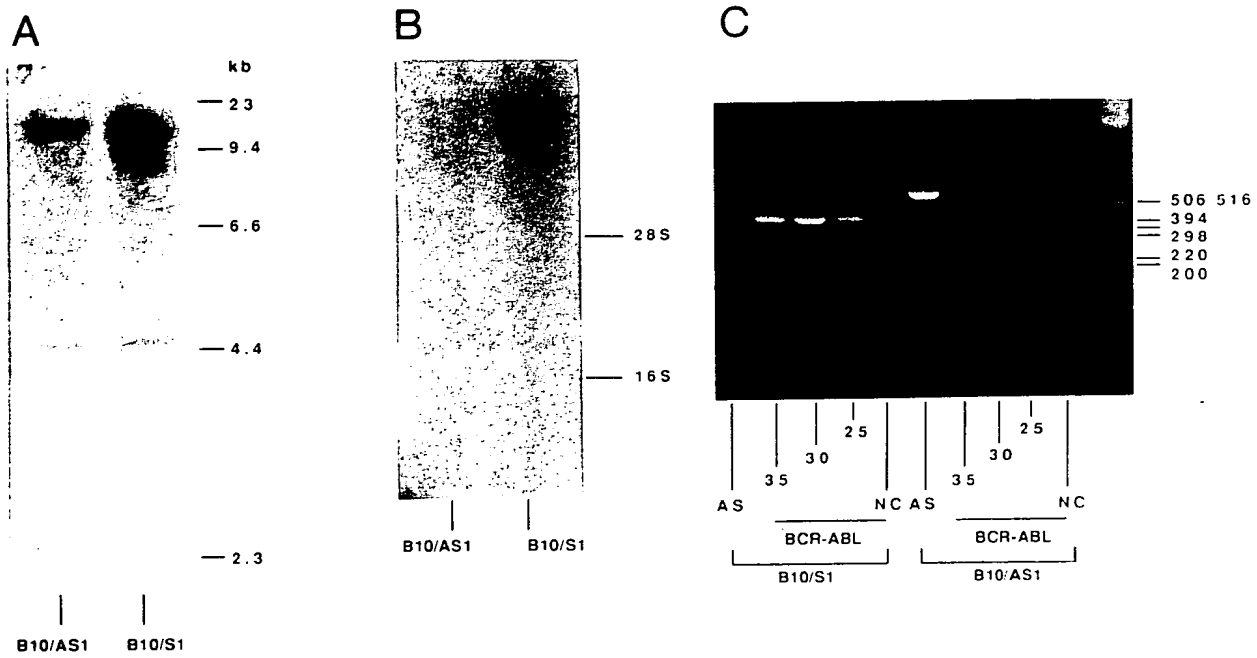

Fig 5. Stable expression in B10 cells: Southern blot, Northern blot, and PCR studies. (A) DNA extracted from B10/AS1 and B10/AS1 digested with *Xba* I and hybridized to the labeled insert INS1. Both lanes show the presence of an 11-kb (BCR-ABL construct) and a 4.5-kb (sense or antisense constructs) band integrated into the B10 cell genome. (B) Total RNA extracted from B10/AS1 and B10/AS1 hybridized to the labeled insert INS2 (that picks up only the BCR-ABL mRNA), showing a strong reduction in the level of BCR-ABL message in B10/AS1 cells. (C) RT-PCR studies on B10/S1 and B10/AS1 cells. The three lanes of 25, 30, and 35 cycles (BCR-ABL) refer to the amplification of the BCR-ABL message in the two types of cells, with NC being a negative control without RT (35 cycles). The two lanes AS show the result of RT-PCR performed with the primers pair amplifying the antisense RNA.

B10 Cells

Transient Expression

Cell proliferation studies. The 5′ antisense transcripts (AS1) were able to specifically inhibit the proliferation of B10 cells in the absence of IL-3, as shown by cell proliferation studies, while not affecting them in IL-3–supplemented culture medium (Fig 3A). This effect on cell growth was maximal between 48 and 72 hours after electroporation. After that period, the growth suppression was released and the B10 cells electroporated with pZip/AS1 started to grow again. The sense construct had no detectable effect on cell kinetics when compared with the original plasmid [pZip/NEO-SV(X)] electroporated using the same experimental protocol.

P210 assay. P210 kinase assays showed an inhibition of the protein expression by more than 90% (Fig 3B) in B10/AS1 cells grown in IL-3–supplemented medium collected at 72 hours. P210 expression returned to normal 96 to 108 hours after electroporation and IL-3 independence was restored by that time. B10/S1 cells did not show any reduction in P210 expression. The second construct (pZ/AS2) had no detectable effect, neither on cell growth nor on P210 expression (Fig 3B).

Stable Transfection

Proliferation studies. In B10 cells expressing the antisense sequences (B10/AS1), independence from IL-3 was completely abrogated (Fig 4A), whereas the cells infected with the sense construct were unaffected. True cell death was observed in B10/AS1 grown without IL-3. Three days after the cells had been transferred to an IL-3–deprived culture medium, no surviving cell could be observed using Trypan Blue and the addition of IL-3 at that stage did not lead to any cell regrowth.

P210 assay. A marked reduction of P210 expression (Fig 3B) was observed in the clones of B10 cells expressing the antisense sequences.

Southern blot studies. In B10/S1 and B10/AS1 DNA digested with *Xba* I (Fig 5A), two bands (approximately 11 and 4.5 kb) are visible, showing that in these cells both the BCR-ABL complete sequences and the sense/antisense sequences are present within the genome.

Northern blot studies. In B10/S1 cells, the 8.5-kb BCR-ABL mRNA can be seen after 2 days of exposure (Fig. 5B), whereas it cannot be detected in B10/AS1. A longer exposure time (15 days) results in the appearance of a faint band in B10/AS1 RNA, showing that the message is present but in much lower amounts in these cells.

PCR studies. After 25 cycles of amplification, the BCR-ABL message is clearly visible, in B10/S1 cells, on the ethidium bromide-stained gel (Fig 5C), and another 5 to 10 cycles do not change significantly the intensity of the band. On the other hand, 30 cycles are needed to clearly identify the mes-

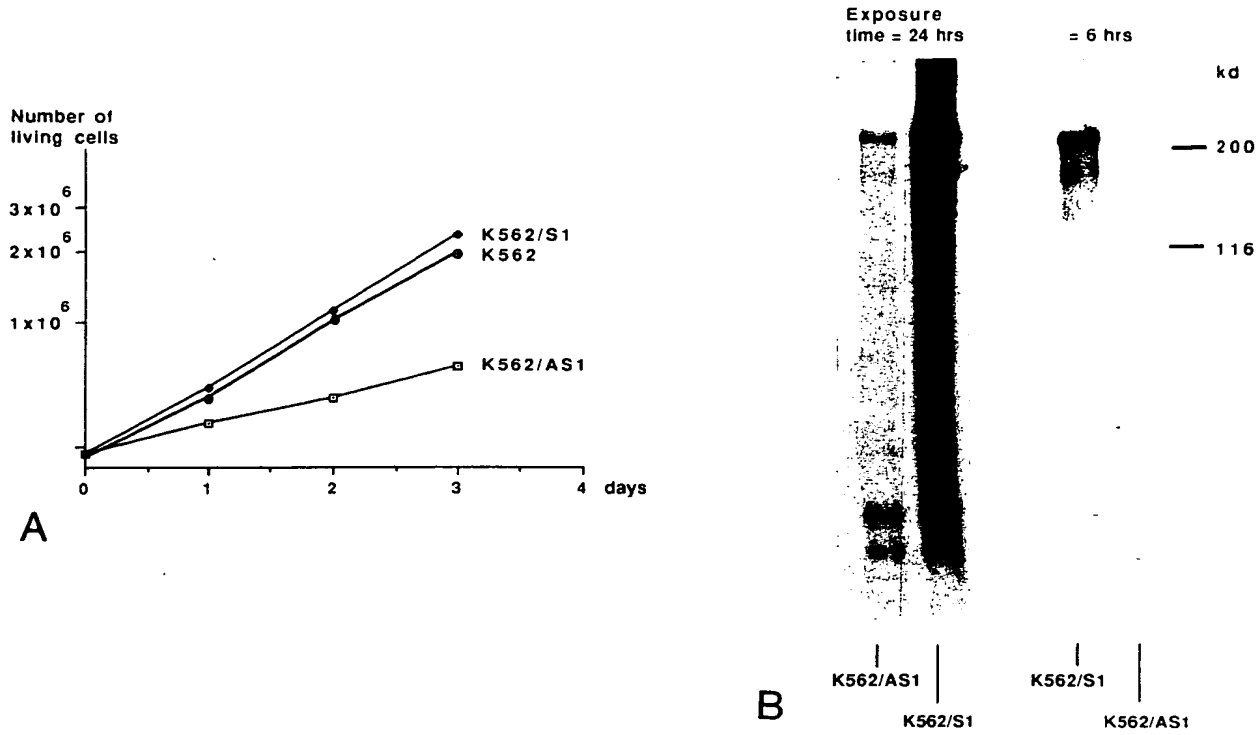

Fig 6. Stable expression in K562 cells: proliferation studies and P210 assay. (A) Comparison of proliferation rate between K562 expressing the antisense RNA (K562/AS1) and the same number of control K562 or K562/S1 growing in the same culture medium. (B) P210 measurement in 4×10^6 K562/S1 cells and K562/AS1 using two different autoradiograph exposure time (6 and 24 hours).

sage in B10/AS1 cells, confirming the results of the Northern blot. This experiment also shows that, despite a decrease in BCR-ABL mRNA within the B10/AS1 cells, the antisense does not lead to complete disappearance of it. As shown by the amplification of the antisense message, BCR-ABL mRNA decrease is seen in parallel with expression of the antisense RNA.

K562 Cells

Proliferation Studies

K562 cells that had integrated the antisense sequences (K562/AS1) showed a reduced growth rate in comparison with control K562 and K562/S1 infected with the sense construct grown in the same culture medium (doubling time: 39.0 ± 2.5, 17.8 ± 2.0, and 18.1 ± 2.3 hours, respectively) (Fig. 6A). This effect on proliferation was not reversed by the addition of IL-3 to the culture medium.

P210 Assay

In K562/AS1, P210 expression was markedly reduced (Fig 6B) when compared with K562/S1 and normal K562 (not shown).

Western Blot Studies

Experiments performed to look at the normal BCR gene products showed a complete disappearance of P160^{BCR} and P130^{BCR} in K562/AS1 (Fig 7) and in HeLa/AS1 cells. No differences were observed in the growth characteristics of HeLa or HL60 cells infected with the antisense construct, showing that the inhibition of the BCR proteins may not be responsible for this change in proliferation rate, inasmuch as conclusions obtained on two cell lines (HL60 and HeLa) can be applied to a third one (K562).

DISCUSSION

We have shown in this report that almost complete inhibition of P210 expression was achievable, in a stable manner, using retrovirally transduced antisense sequences targeted against the 5′ portion of the hybrid BCR-ABL mRNA. This effect could be obtained in cells expressing the BCR-ABL mRNA at a much higher level than what can be expected in fresh CML cells.[18] If successfully achieved in CML-derived cells, this inhibition might have caused the cells having integrated the antisense sequences to die, thus preventing us to verify our hypothesis. This is why we chose as a first target the B10 lymphoid cell line (previously rendered IL-3 independent upon expression of P210-coding sequences). In the event of inhibition of P210 expression, which would lead to cell death in a culture medium deprived of IL-3, the cells can be rescued by addition of exogenous IL-3, thereby allowing us to assay P210 expression on living material. Their relative resistance to retroviral infection led us to first set up a transient

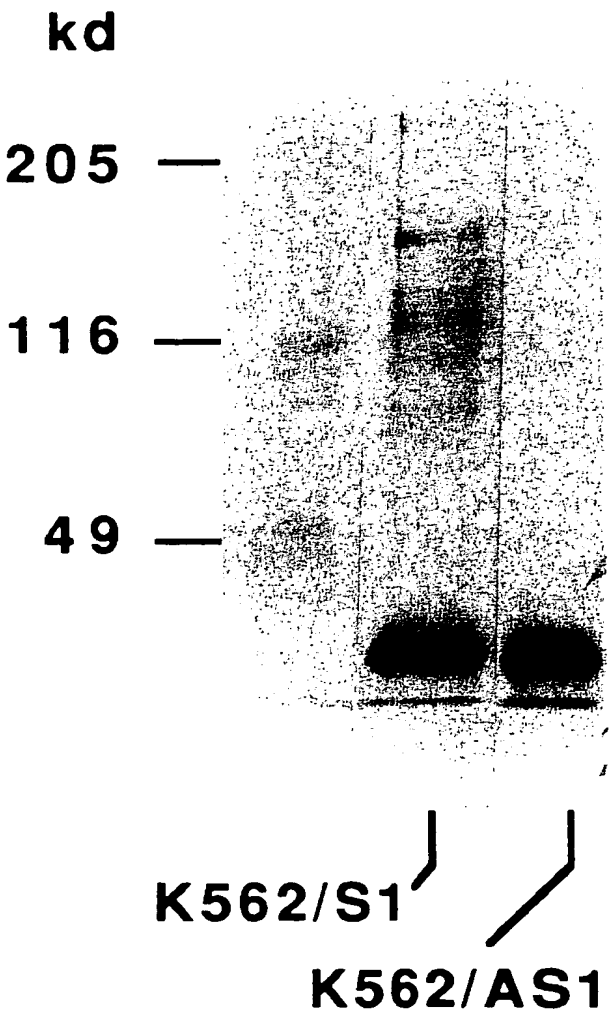

Fig 7. Stable expression in K562: Western blot studies. Comparison of K562/S1 and K562/AS1 cells showing inhibition of $P210^{BCR-ABL}$ and $P160^{BCR}$-$P130^{BCR}$ in K562/AS1.

expression assay that ultimately proved a very useful tool for evaluating the effect of a construct without having to go through each time the long procedure of making packaging cells, cloning them, and testing the supernatants. Our experience with antisense oligonucleotides[12] led us to first use the sequences complementary to the 5' end of BCR/ABL mRNA before designing junctional antisense constructs that would have the theoretical advantage of leaving unaffected the normal BCR gene product. The 5' antisense transcripts, generated under the control of MoMLV LTR promoter sequences and stably expressed in B10 cells, were able to specifically kill them in the absence of IL-3, as shown by cell proliferation studies, and to deeply inhibit P210. Northern blot studies showed that one mechanism for inhibition of P210 expression is a degradation of the BCR-ABL mRNA. However, PCR experiments showed persistence of some BCR-ABL message, but because inhibition can also occur at the translational level, a further block can account for almost complete blockade of P210 synthesis. The P210 assay performed on K562 expressing the antisense sequences showed a dramatic inhibition of P210 expression; this occurred despite known amplification of the BCR-ABL gene in K562 cells. It is worth mentioning that the K562/AS1 cells that were investigated at different moments showed slight variations in the level of inhibition, ranging from no P210 detectable to a small amount of it being picked up by the assay (the latter case being shown in Fig 6). The fact that these cells are not clonal may account for variation in the expression of the antisense transcripts according to the integration site within the genome. We chose to show an experiment that represents one of the weakest inhibitions we found to not overinterpret the data, but all the P210 assays were at least as good in terms of inhibition, with some of them showing no detectable protein. We may thus speculate that a construct capable of inhibiting P210 to such an extent should be very active in fresh CML cells. We think that the change of the proliferation rate of the K562/AS1 cell is related to P210 inhibition rather than to inhibition of the normal BCR products, because HeLa and HL60 cells, in which inhibition of the BCR gene has occurred, have the same growth characteristics as the cells expressing the sense sequences. However, inhibition of the normal BCR gene may have different effects in different cell lines, and only specific inhibition of P210 with a junctional antisense construct will allow us to answer that question definitively. Our first attempt to design a junctional antisense construct failed to inhibit P210 expression. We have no data allowing us to understand why this junctional construct had no effect. It was also efficiently transcribed in 3T3 fibroblasts and the lack of effect cannot therefore be attributed to a lower transcription efficiency. It is possible that secondary structures of the hybrid BCR-ABL and/or the antisense RNAs make these junctional sequences less likely to hybridize. However, antisense strategies are still, to a large extent, empirical and further experiments with different types of junctional constructs are under way. These results also show that survival of a cell line derived from a CML blast crisis (K562) is possible despite reduction of P210 expression. We cannot tell from our experiments whether a low level of P210 expression is sufficient to keep the cells alive, or whether other mechanisms play an essential role when P210 is inhibited. The fact that the changes induced in K562 growth characteristics was not reversed by the addition of IL-3 to the medium indicates that P210 must exert its action in CML cell lines via an IL-3–unrelated pathway. Although apparently less dramatic than results obtained on CML cells in bone marrow culture using antisense oligonucleotides[13] in which inhibition of the Philadelphia-positive compartment occurred upon inhibition of BCR-ABL mRNA, our results may be explained by the fact that a leukemic cell line may have acquired secondary characteristics, making it less dependent on a single genetic event that fresh CML cells do not possess. Further experiments on fresh CML cells in culture will be needed to see whether infection of these cells with antisense constructs, followed by antibiotic selection, will lead to eradication of Philadelphia-positive cells

or simply to the persistence of these cells, despite abolition of P210 expression, with possible modifications of their leukemic phenotype (Philadelphia-positive, P210-negative cells).

In conclusion, we feel that the use of retroviral antisense sequences may prove useful for research and therapeutic use. K562 cells with stable inhibition of P210 expression may be used to study further the mechanisms by which the effects of the inhibition could be reversed. Furthermore, our results suggest an approach to in vitro gene therapy in CML: Philadelphia-negative or even Philadelphia-positive, P210-negative hematopoiesis might be restored by autografting patients with bone marrow cells that had been incubated with the antisense supernatant, followed by a selection procedure to ensure that all reinfused stem cells had integrated antisense sequences.

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Inhibition of Human Immunodeficiency Virus Type 1 Replication in Human T Cells Stably Expressing Antisense RNA

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Human T-lymphoid cell lines with constitutive intracellular expression of antisense RNA complementary to a 407-bp sequence of the 5′ leader-*gag* region of human immunodeficiency virus type 1 were established by using a nonretroviral expression vector. In cell lines with antisense RNA expression detectable by Northern (RNA) hybridization, human immunodeficiency virus type 1 replication was inhibited to 88% 10 days postinfection and this inhibition lasted 3 weeks postinfection.

Antisense nucleic acids have been used successfully to inhibit specifically the expression of cellular genes (for reviews, see references 22, 24, and 25). Viral gene expression or other essential steps in the viral life cycle can also be affected by antisense nucleic acids (20, 28). This has been demonstrated for procaryotic viruses (7) as well as for eucaryotic viruses including retroviruses. Thus, replication of the Rous sarcoma virus was partially suppressed by antisense RNA expressed from eucaryotic vectors (6, 23), and the replication of human immunodeficiency virus type 1 (HIV-1) could be reduced by antisense oligodeoxyribonucleotides (1, 9), chemically modified antisense oligodeoxyribonucleotides (5, 10, 21, 27), and chemically modified antisense oligoribonucleotides (19) added to the culture medium.

In contrast to antisense oligonucleotides being applied extracellularly, intracellularly expressed antisense RNA is a potential antiviral agent which might be used for the development of "intracellular immunization" against virus infection (2). This is also indicated by the finding that HIV-1 replication can be inhibited temporarily when infectious proviral HIV-1 DNA is comicroinjected with an HIV-1 antisense RNA expression plasmid into human cells (18).

In this work, we show that HIV-1 replication is reduced in human T-cell lines constitutively expressing HIV-1 antisense RNA targeted against 407 bp of the 5′ leader-*gag* region. This result indicates that intracellularly expressed antisense RNA is a potential anti-HIV-1 agent.

Expression vectors. The expression plasmid pKEX-1, as well as the plasmid vectors p2as and p2s designed for the expression of HIV-1 antisense and sense RNA, respectively (Fig. 1A) have been described elsewhere (18). Briefly, constitutive transcription is directed by the human cytomegalovirus immediate-early promoter-enhancer element (4). The HIV-1 fragment expressed in p2as and p2s was derived from the HIV-1 proviral clone pBH10 (14) as a 407-bp *Sac*I-*Hin*dIII fragment (positions 222 to 629) covering 112 nucleotides of the 5′ leader and 295 nucleotides coding for the *gag* 5′ region. This sequence also includes the first splice donor, which is used in all known spliced HIV-1 transcripts (11, 17). The resulting transcripts are terminated by 3′-processing signals from the simian virus 40 early region (12) and have a total expected length of 1,120 nucleotides. Additionally, the hygromycin B resistance gene (3), in *cis* on all plasmids, serves as a dominant selection marker.

Cell lines with constitutive antisense-sense RNA expression. The human $CD4^+$ T-lymphoid cell line Jurkat (16, 26) was used as a tissue culture model system for measuring the effects of intracellular HIV-1 antisense RNA expression on HIV-1 replication. For the generation of stably transfected clones, Jurkat cells were electroporated in the presence of one of the plasmids pKEX-1, p2as, and p2s under conditions essentially as described previously (8) except for the initial voltage (250 V). Under hygromycin B selection (500 μg/ml) in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5% CO_2, resistant clones arose from total viable transfected cells with a frequency in the range of 5×10^{-6}. The morphology of the stable Jurkat clones was indistinguishable from that of the parental cells, and all cell lines exhibited the same proliferation rate. The expression levels of the T-cell-specific surface antigens OKT4a, OKT4, and OKT8 as measured by radioimmunoassay also were indistinguishable for the parental cell line Jurkat, one representative (i.e., inhibitory) p2as clone, and one p2s clone.

The Jurkat clones were analyzed for the status of the transfected recombinant plasmid DNAs by restriction of chromosomal DNA and subsequent Southern hybridization with ^{32}P-labeled plasmid DNA. Approximately 90% (25 of 28) transfectants analyzed integrated the plasmid DNA into their chromosome as a single copy. In most cases, this occurred without rearrangements in regard to the expression cassette for antisense and sense transcription, which is shown for four representative p2as and p2s clones in Fig. 1B. Northern (RNA) hybridization of total cytoplasmic RNAs from 14 independent clones which had been positive for integrated plasmid DNA showed detectable amounts of HIV-1 sequence containing transcripts in 9 cases (64%). The apparent size of p2as-derived HIV-1 antisense transcripts on Northern blots (Fig. 1C) was approximately 50 nucleotides greater than that of the sense RNA transcribed from p2s, and also, the antisense transcripts appeared to be less abundant.

HIV-1 replication in antisense RNA-expressing cells. Since clonal variation was expected in the course of HIV-1 infection of different cell clones, to exclude clonal differences unrelated to the antisense RNA expression, HIV-1 infection was investigated in a total number of 10 independent stable p2as transfectants and was compared with infection in 10

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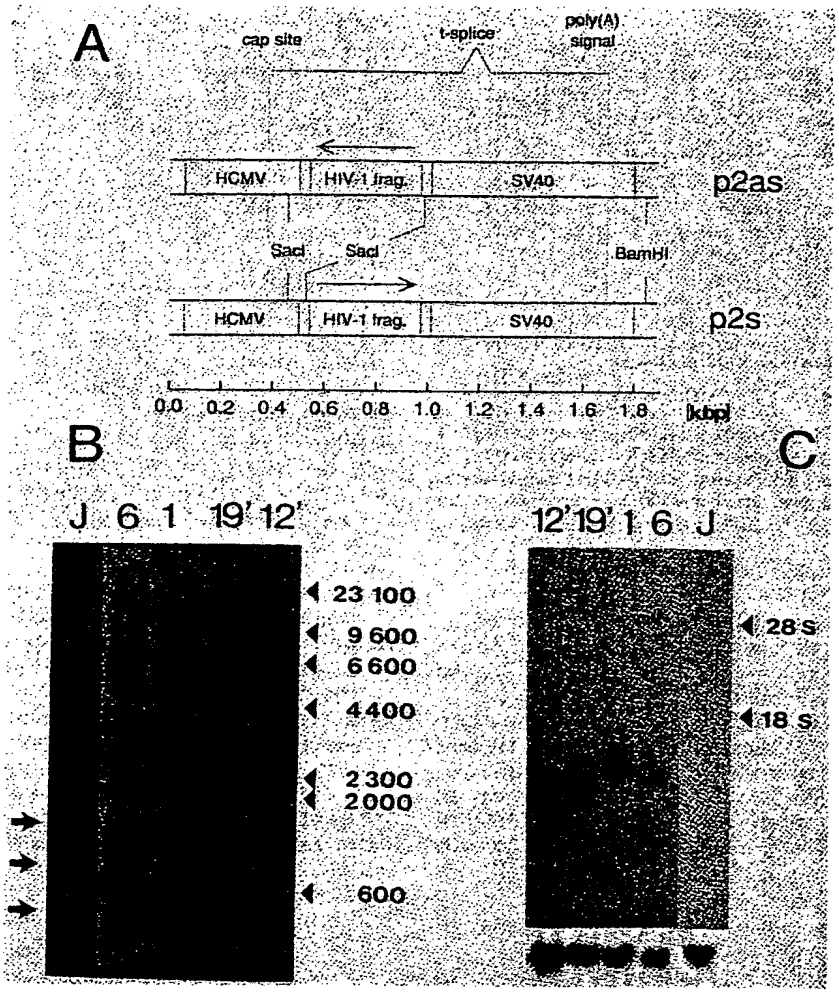

FIG. 1. Chromosomal integration and expression of HIV-1 antisense RNA and sense RNA in stably transfected Jurkat clones. (A) Schematic representation of the expression cassettes for antisense RNA (p2as) and sense RNA (p2s) transcription. The derived transcripts (above) contain 54 bp of the human cytomegalovirus (HCMV) immediate-early promoter-enhancer fragment downstream from the transcriptional start site followed by 407 bp of HIV-1 5′ leader-*gag* sequence and the t-splice and polyadenylation signals from simian virus 40 (SV40). The approximate distances between the restriction sites used in Southern analysis (*Sac*I and *Bam*HI) can be seen with the scale indicated below. (B) Autoradiogram from Southern hybridization of *Sac*I-*Bam*HI-restricted chromosomal DNA (10 μg) of two p2as clones (lanes 1 and 6), two p2s clones (lanes 12′ and 19′), and the parental cell line Jurkat (lane J). DNAs were separated on 0.8% agarose gel, blotted onto Gene Screen Plus membrane, and hybridized with ^{32}P-labeled plasmid p2s under stringent conditions (hybridization, 50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 42°C; washing, 2× SSC, 0.1% sodium dodecyl sulfate, three 10-min washes, 68°C). In addition to two flanks from the single-copy integration in each clone, the relatively small internal *Bam*HI-*Sac*I fragments indicate the difference between p2as and p2s clones. The arrows on the left indicate positions of the 1,200-bp *Bam*HI-*Sac*I fragment from p2s clones and the 800- and 400-bp fragments, respectively, in p2as clones. (C) Autoradiogram from Northern hybridization of total cytoplasmic RNA (10 μg) isolated from p2as clones (lanes 1 and 6), p2s clones (lanes 12′ and 19′), and parental Jurkat cells (lane J). RNAs were separated by formaldehyde-agarose gel electrophoresis, blotted onto Gene Screen Plus membrane, and hybridized with ^{32}P-labeled plasmid p2s under stringent conditions (hybridization and washing as described above). Hybridization signals at the same positions as shown here were seen with ^{32}P-labeled strand-specific riboprobes, additionally distinguishing between the antisense and sense transcripts. As an internal probe for the relative amounts of RNA in each sample, the blot was hybridized with a human β-actin cDNA probe (bottom part of the figure).

independent Jurkat(pKEX-1) clones as well as eight independent Jurkat(p2s) clones.

Jurkat clones were infected with cell-free supernatants from HIV-1-infected human T-cell lines (H9 or Jurkat; HIV-1 strain HTLV-IIIB [13]). Since the spread of HIV-1 in human T cells was to be measured, rather than the initial infection efficiency immediately after the addition of infectious virus to the cells, a low dose of infectivity was used. The initial infectious dose generated approximately 1% HIV-1 antigen immunofluorescence-positive cells 10 days postinfection in pKEX-1 and p2s clones as well as in the parental cell line Jurkat. The replication of HIV-1 in Jurkat clones was monitored by two assays: enzyme-linked immunosorbent assay (ELISA) measurements of HIV-1-specific antigens released into the culture supernatants, indicating production of free virus particles; and the percentage of

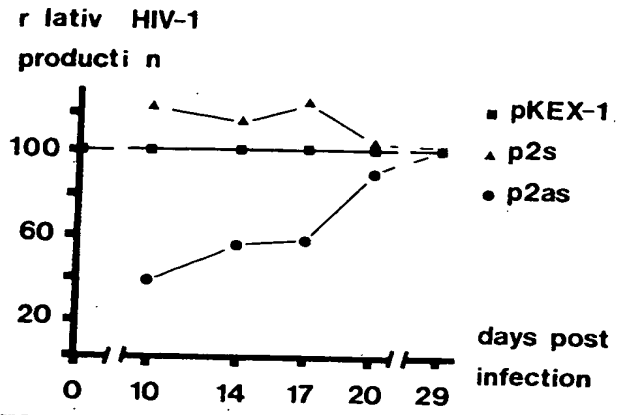

FIG. 2. Time course of HIV-1 virus production as the average of 9 independent p2as clones relative to 10 pKEX-1 clones and 6 p2s clones. The HIV-1 replication is standardized to pKEX-1 clones (100). HIV-1 antigen concentrations in cell-free supernatants were measured by a commercial ELISA system (Organon Teknika, Boxtel, The Netherlands).

cells expressing HIV-1 antigens, which was determined by indirect immunofluorescence with serum from a Tanzanian patient with AIDS and a fluorescein isothiocyanate-linked goat anti-human immunoglobulin antiserum.

During the first 14 days after infection, HIV-1 antigen concentrations in the culture supernatants from p2as clones were reduced in comparison with those of pKEX and p2s control clones, with a maximum of 65% reduction on day 10 postinfection (Fig. 2). Two weeks after infection, control clones showed 50 to 80% immunofluorescence-positive cells and the HIV-1 antigen concentration in cell-free supernatants was in the range of 2×10^4 units (Fig. 3). In Jurkat(p2as) clones, spread of infection is slower and reaches the same maximal values 3 to 4 weeks postinfection (data not shown). This delay in the spread of HIV-1 infection in Jurkat(p2as) clones suggested a transient inhibition of HIV-1 replication in the first period of infection (2 weeks).

For a more detailed analysis, more individual HIV-1 infection experiments of p2as, pKEX-1, and p2s clones were performed. In addition, the group of p2as clones was subdivided into those clones with detectable amounts of cytoplasmic HIV-1 antisense RNA [p2as(RNA^+)] by Northern hybridization and those clones with no detectable cytoplasmic HIV-1 antisense RNA [p2as(RNA^-)]. The p2as(RNA^+) clones showed an inhibition of virus release into the culture medium which was 88% on day 10 and 68% on day 14 postinfection in comparison with pKEX-1 clones (Fig. 3A and B). On day 14 postinfection, in p2as(RNA^+) cultures there were 5% immunofluorescence-positive cells compared with 27% in pKEX-1 controls, which is a fivefold reduction (Fig. 3C). Indirect immunofluorescence in this experiment could not be quantitated exactly on day 10 postinfection because of the low numbers of infected cells. However, the percentage of positive cells in pKEX-1 controls was equal to or smaller than 1% and was below 0.1% in p2as(RNA^+) clones.

The experiments described above showed that $CD4^+$ cells from the human T-cell line Jurkat with constitutive HIV-1 antisense RNA expression targeted against 407 bp of the 5' leader-*gag* region of HIV-1 show reduced viral replication. This was measured with a total number of 10 independent p2as clones in comparison with control clones, i.e., clones from the same human T-cell line which have been stably

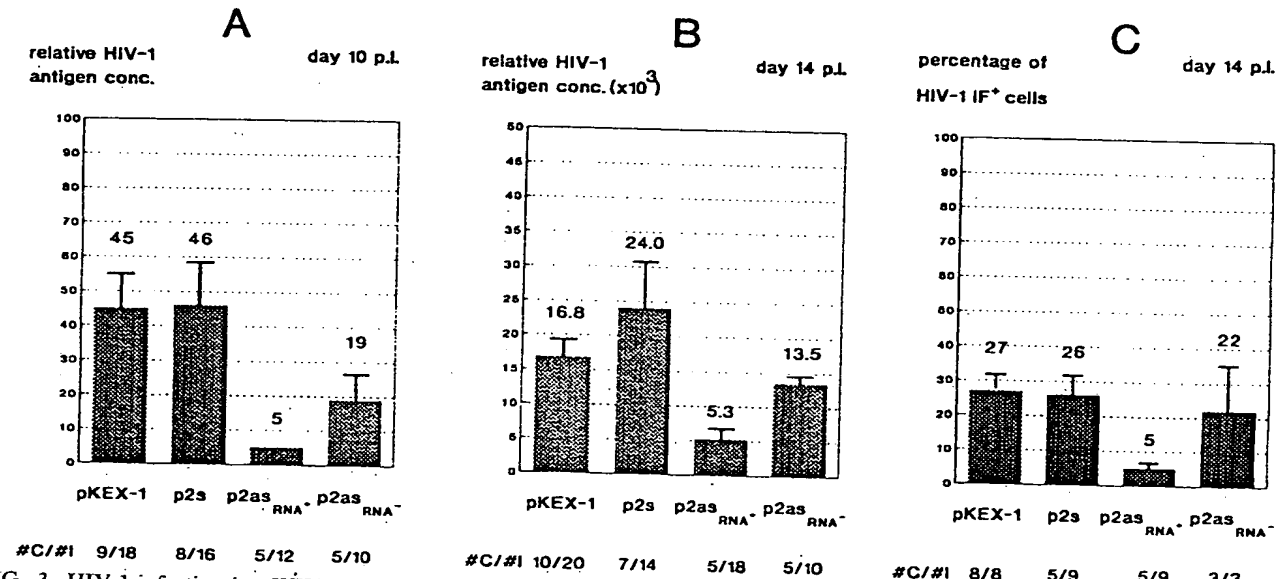

FIG. 3. HIV-1 infection in pKEX-1, p2s, and p2as clones. The p2as group was subdivided into clones with antisense RNA expression [p2as(RNA^+)] and clones without detectable antisense RNA expression [p2as(RNA^-)] as measured by Northern hybridization. Numbers below the bars of each group indicate the number of individual clones (#C) and the number of independent infection experiments within the group (#I). The error bars indicate the standard error of the mean. (A and B) Comparison of the release of cell-free virus, i.e., relative antigen concentrations in HIV-1-infected pKEX-1, p2s, p2as(RNA^+), and p2as(RNA^-) clones measured by ELISA on days 10 and 14 postinfection. There is no error bar for p2as(RNA^+) in panel A since the relative antigen concentration of 5 is the greatest estimate for this value, i.e., the true value is equal to or smaller than 5. (C) Percentage of HIV-1-infected cells on day 14 postinfection as measured by indirect immunofluorescence.

transfected with the original expression plasmid pKEX-1 or the corresponding sense RNA expression vector p2s by using the same transfection and selection conditions. This indirect evidence for the involvement of antisense RNA in the antiviral effect is further supported by the observation that p2as clones with undetectable antisense RNA expression in Northern analysis [p2as(RNA^-) clones] show reduction of virus replication to a clearly smaller extent in comparison with p2as(RNA^+) clones (Fig. 3).

The inhibition of HIV-1 replication in human $CD4^+$ cells by intracellular antisense RNA expression, although incomplete and with variations among different clones, suggests that antisense RNA could be used as an antiretroviral agent. Inhibition of HIV-1 replication had also been observed previously in a transient comicroinjection assay with the same antisense RNA expression plasmid p2as (18).

The reason why HIV-1 antisense RNA-expressing cells are protected in early stages of HIV-1 infection (i.e., delay in spread of HIV-1 infection) but become infected in later stages with similar virus proliferation rates as in pKEX-1 clones is unknown. However, the fact that finally (2 weeks postinfection) p2as(RNA^+) cultures were also infected was not due to reduced antisense RNA expression. Even after 3 weeks of HIV-1 infection, p2as(RNA^+) cells (40 to 70% HIV-1 antigen immunofluorescence-positive cells) did not show a detectable difference in antisense RNA expression levels as measured by Northern analysis (data not shown).

The sense RNA expression vector (p2s) in stably transfected cells did not have an inhibitory effect on HIV-1 replication, whereas it inhibited HIV-1 replication to the same extent as did p2as when comicroinjected together with infectious proviral HIV-1 DNA into human cells (18). This differential behavior of p2s might be due to differences of both test systems. In the transient comicroinjection assay with human epithelioid cells, probably other steps in the replication cycle of HIV-1 are affected, as in T cells with constitutive RNA expression, in which all steps of the viral life cycle involving single-stranded viral nucleic acids are potential targets. Recently, intracellular expression of ribozyme-containing RNAs has been used to inhibit HIV-1 replication in stably transfected $CD4^+$ HeLa cells (15). The inhibitory effect in this case (95 to 97.5%) was measured 7 days after HIV-1 infection and thus seems to be in a range of inhibition similar to that measured in this work for antisense RNA.

Future improvements in the antiviral activity of intracellularly expressed antisense RNA will involve prolongation of the inhibitory effect as well as increase of the antisense effect. The latter could be achieved by the use of more effective HIV-1 target regions and might be supported by ribozyme sequences inserted into antisense transcripts.

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ADDENDUM IN PROOF

After submission of this note an article was published (A. Rhodes and W. James, J. Gen. Virol. 71:1865–1974, 1990) in which similar results were obtained with Jurkat cells and a retroviral vector system expressing HIV-1 *tat* antisense RNA.

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RESEARCH ARTICLE

Constitutive and Conditional Suppression of Exogenous and Endogenous Genes by Anti-Sense RNA

Jonathan G. Izant and Harold Weintraub

In order to study the biological functions associated with cloned gene sequences, we previously designed a strategy for specifically inhibiting their expression in vivo (*1*). We used, as a test system, the herpes simplex virus (HSV) thymidine kinase (TK) gene in plasmid DNA constructions designed to transcribe the anti-sense (noncoding) DNA strand. The anti-sense transcript has a sequence complementary to the target messenger RNA (mRNA) and can presumably anneal with the mRNA and disrupt normal processing or translation. The anti-sense TK plasmid is constructed in vitro by inverting the TK protein coding sequence with respect to its promoter. Such a plasmid will specifically inhibit expression of the cognate sense TK plasmid after both plasmids are microinjected into LTK$^-$ cells (*1*). The promising results with the HSV-TK model system suggest that anti-sense RNA can provide an additional methodology for genetic analysis in eukaryotic systems that are not readily-amenable to standard mutational analysis. Inhibition of function by anti-sense RNA is a regulatory strategy in prokaryotes where it has been found to control translation (*2*) as well as the activity of RNA primers for initiating episome DNA replication (*3*). Similar mechanisms have not yet been described in eukaryotes.

We have now extended the use of anti-sense inhibition to both transient and stable DNA-mediated transformation systems. We show that a fragment as short as 52 bases of 5′ untranslated anti-sense TK RNA inhibits TK activity. The inhibition is sequence specific. Anti-sense herpes TK inhibits sense herpes TK, but not expression from the non–cross-hybridizing chicken TK gene, while anti-sense chicken TK inhibits expression from a sense chicken TK plasmid, but not from a sense herpes TK plasmid. Conditional, dexamethasone-inducible, anti-sense inhibition is demonstrated by the use of the long terminal repeat (LTR) of the murine mammary tumor virus (MMTV) to direct the synthesis of anti-sense TK RNA. We show that a stably introduced TK gene is also inhibited by anti-sense TK, and finally that expression of the normal end genous cytoplasmic actin gene can be inhibited by anti-sense actin expression plasmid constructions. The actin inhibition is detected as a diminution of the actin microfilament array and as a decrease in cell viability.

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DNA. Sense TK plasmid (2 μg/ml) plus a 200-fold excess of anti-sense or control plasmid DNA's were microinjected into TK⁻ L-cell nuclei. After a 20-hour incubation period in [³H]thymidine, TK activity was assessed by determining the number of cells incorporating labeled thymidine by autoradiography as a proportion of the cells that survived microinjection.

Plasmids injected	TK cells (%)	Cells injected (No.)
HSV TK plus:		
Anti-sense HSV TK	7.5*	223
Anti-sense 5′ TK	8.3*	216
Anti-sense chicken TK	34.0	190
Control plasmid	41.0	96
Chicken TK fusion gene (Fig. 1b) plus:		
Anti-sense HSV TK	38.0	170
Anti-sense 5′ TK	0.5*	188
Anti-sense chicken TK	17.0*	230
Control plasmid	35.0	203

*All cells with silver grains (detectable enzyme activity) were scored as TK⁺. As in Fig. 3, however, analysis of autoradiographic grain density reveals a four- to fivefold suppression of TK activity by anti-sense plasmid DNA so that the actual inhibition is greater than that suggested by merely assaying the proportion of TK⁺ cells (*1*).

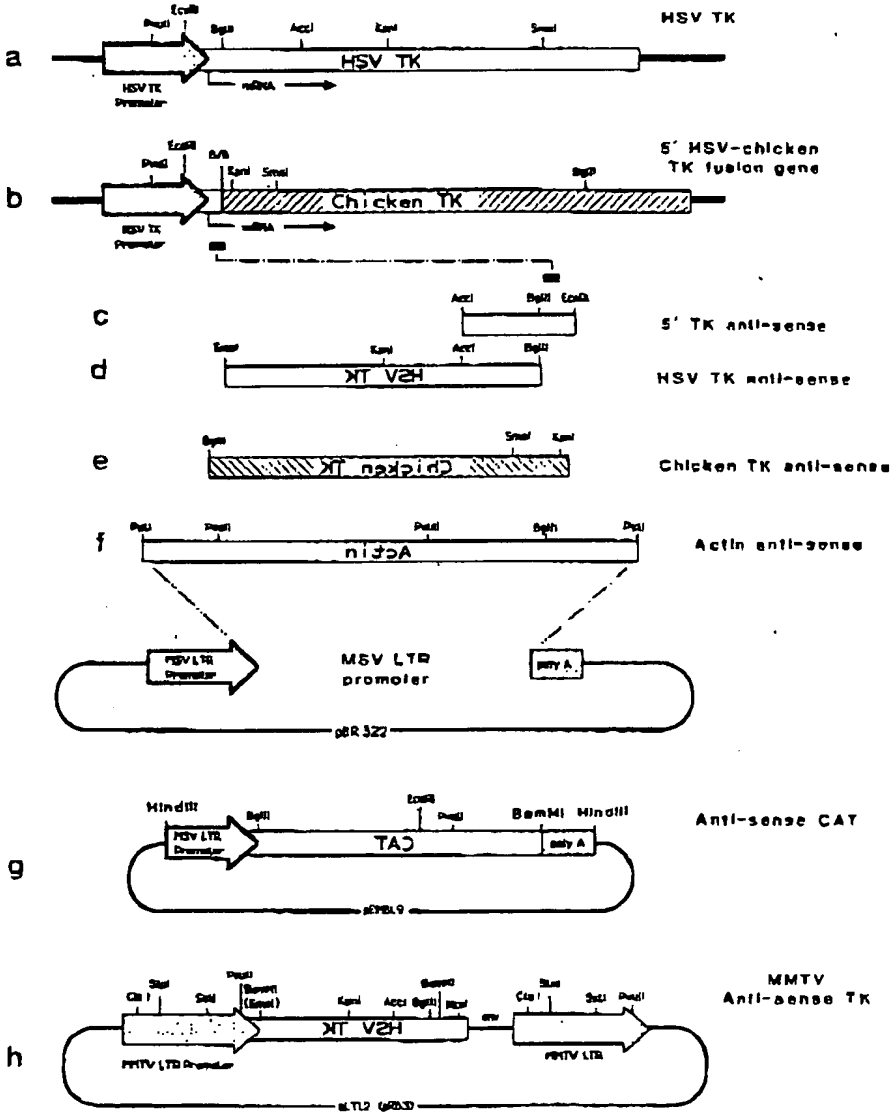

Fig. 1. Plasmid DNA's used in this study (*16*). (a) The wild-type HSV-TK plasmid, which uses its own promoter. (b) The chicken TK structural gene was fused in the sense orientation to the HSV-TK promoter sequence corresponding to 52 bp of 5′ untranslated DNA. (c to f) Various gene restriction fragments were cloned in flipped orientation into a pBR322 derived vector containing the MSV LTR promoter and an SV40 early transcript polyadenylation signal. The black box represents the 52 bases in common between the 5′ TK anti-sense (c) and the 5′ region of HSV-TK chicken TK fusion gene (b). B/B is a Bam HI. Bgl II ligation site. (g) Anti-sense CAT gene plasmid. This plasmid contains the CAT gene coding sequence inserted between an MSV-LTR promoter and an SV40 poly A site which has been cloned in the EMBL vector (*14*). (h) Murine mammary tumor virus LTR promoter. This hormone inducible anti-sense TK plasmid is derived from pLTL-2 (*6*) and contains an anti-sense HSV-TK coding sequence inverted next to the left-hand MMTV LTR promoter. Plasmid DNA's were constructed, produced, and purified as described (*1*).

plasmid designed to transcribe a 1364-base anti-sense TK RNA complementary in sequence to the normal sense TK mRNA from base +51 to +1415 (relative to the transcription initiation site) specifically inhibits expression of a sense TK plasmid in mouse L cells (*1*). To determine the amount of sequence required for anti-sense inhibition of herpes TK, we flipped a TK gene fragment spanning the region between −80 (an Eco RI site) and +343 (an Acc I site) and inserted this sequence between the MSV LTR promoter and the SV40 polyadenylation site in inverse orientation (Fig. 1c). Co-injection of this DNA (termed the 5′ TK anti-sense plasmid) with the normal sense herpes TK plasmid (Fig. 1a) at a ratio of 200:1 (anti-sense to sense) gave marked inhibition of TK activity as determined by incorporation of ³H-labeled thymidine (Table 1).

To test whether this 5′ anti-sense plasmid could inhibit a gene with even less complementarity, we used an HSV-TK fragment containing the promoter sequences and 52 bp of 5′ untranslated RNA ligated to the chicken TK structural gene. This fusion gene construction (Fig. 1b) gives normal levels of TK activity after microinjection into the nucleus. Since the herpes and chicken TK genes do not cross-hybridize, RNA transcripts from the 5′ TK anti-sense plasmid are complementary to only the first 52 bases of 5′ untranslated HSV-TK sequence present in the chimeric mRNA (black boxes adjacent to Fig. 1, b and c). The 5′ TK anti-sense plasmid transcript does not overlap the initiator AUG (A, adenine; U, uracil; G, guanine) codon of the

fusion gene transcript and is not complementary to the protein coding domain. When the two plasmids were co-injected into LTK$^-$ cells at a ratio of 200:1 (anti-sense to sense), complete suppression of TK activity was observed (Table 1 and Fig. 2). Inhibition was significantly greater than that observed in the initial anti-sense experiments (*1*) which used an anti-sense vector covering the protein-coding domain of HSV-TK downstream of the Bgl II site. Hence anti-sense RNA complementary to the region upstream of the Bgl II site as well as regions downstream of Bgl II will inhibit TK activity.

The 5′ anti-sense herpes TK plasmid failed to inhibit expression from the wild-type chicken TK gene as would be expected. However, an anti-sense chicken TK construction (Fig. 1e) does give a four- to fivefold reduction of TK activity as assayed by autoradiographic grain density after injection of the sense chicken TK plasmid (Fig. 3). Thus, chicken TK is inhibited by anti-sense chicken TK, but not anti-sense herpes TK while herpes TK is inhibited in a reciprocal fashion by anti-sense herpes TK, but not anti-sense chicken TK plasmids. This provides additional evidence for the sequence specificity of anti-sense plasmid inhibition of gene activity.

Inhibition of TK colony formation by anti-sense DNA transfection. In the aforementioned studies as well as our previous report (*1*) anti-sense DNA was microinjected directly with glass micropipettes into cell nuclei. For many purposes, less technically demanding methods, such as DNA mediated anti-sense transformation, would be useful. To test whether anti-sense DNA vectors would work in transfection systems, 100-mm plates of LTK$^-$ cells were transfected with 50 ng of herpes TK plasmid, 20 μg of carrier calf thymus DNA, and from 0 to 5 μg of anti-sense or control DNA. The number of TK-positive colonies was then assayed (Table 2). Control cultures yielded from 250 to 500 colonies per dish, while cultures that received 5 μg of anti-sense TK DNA (a 100-fold excess of anti-sense to sense DNA) had 10 to 20 times fewer colonies (Fig. 4). In some experiments, as little as a tenfold excess of anti-sense TK gave similar degrees of inhibition. Reproducible inhibition was not apparent at a 1:1 ratio. We suspect that the residual TK-positive colonies that emerge at high levels of anti-sense TK represent the small proportion of transfected cells that fail to take up or express the co-transforming anti-sense DNA (*4*). When pBR322 (5 μg per dish) was used as a control, there was a no-

Abstract. *Plasmid DNA directing transcription of the noncoding (anti-sense) DNA strand can specifically inhibit the expression of several test genes as well as normal, endogenous genes. The anti-sense plasmid constructions can be introduced into eukaryotic cells by transfection or microinjection and function in both transient and stable transformation assays. Anti-sense transcripts complementary to as little as 52 bases of 5′ untranslated target gene mRNA specifically suppress gene activity as well as, or more efficiently than, anti-sense transcripts directed against the protein coding domain alone. Conditional anti-sense inhibition is accomplished with the use of hormone-inducible promoter sequences. Suppression of endogenous actin gene activity by anti-sense RNA is detected as a decrease in growth rate and as a reduction in the number of actin microfilament cables. These observations suggest that anti-sense RNA may be generally useful for suppressing the expression of specific genes in vivo and may be a potential molecular alternative to classical genetic analysis.*

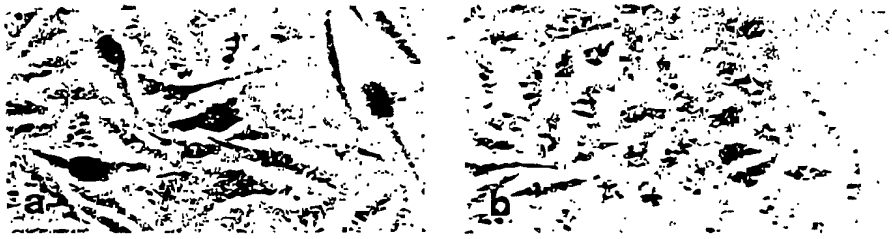

Fig. 2. Autoradiography of cells co-injected with TK genes and 5′ TK anti-sense DNA. Cells were injected with the chimeric HSV promoter chicken TK fusion gene (Fig. 1b) and (a) a control anti-sense β-galactoside plasmid or (b) a 200-fold excess of 5′ TK anti-sense plasmid (Fig. 1c). Thymidine labeling and autoradiography were performed as described (*1*). No significant signal is detectable in the cells co-injected with the anti-sense 5′ TK (×500).

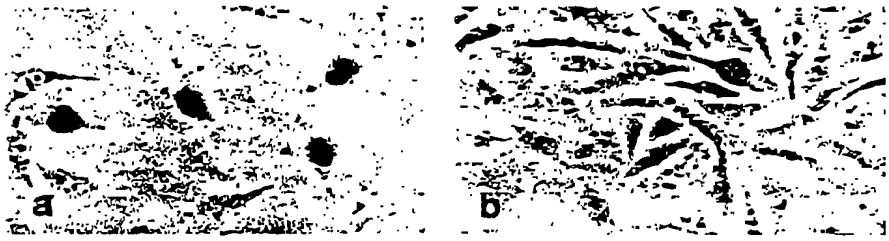

Fig. 3. Anti-sense chicken TK inhibits chicken TK. Representative micrographs of cells co-injected with a chicken TK plasmid and a 200-fold excess of a control herpes TK anti-sense plasmid (a) or a similar excess of anti-sense chicken TK plasmid DNA (b) (×500).

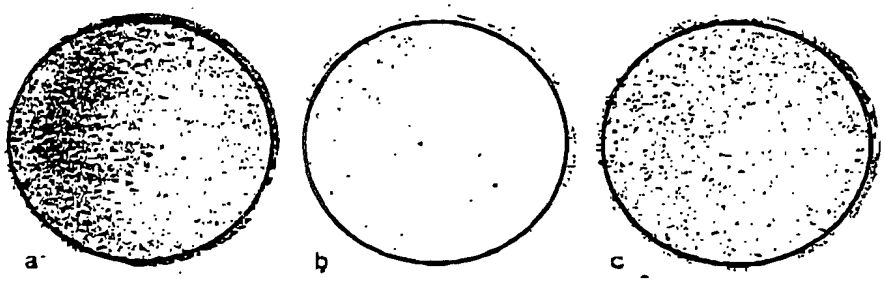

Fig. 4. Inhibition of herpes TK colony formation. LTK$^-$ cells were co-transformed with herpes TK (50 ng) and either 5 μg of anti-sense chicken TK (a) or 5 μg of anti-sense herpes TK (b) (*4*). Colonies were stained after 2 weeks in HAT medium. The inhibition by anti-herpes TK could be overcome by including a sense chicken TK plasmid (50 ng) in the transformation mix (c). All transformations contained 20 μg of carrier calf-thymus DNA.

quency, but this was marginal compared to the reproducible and more dramatic decreases observed with the anti-sense TK DNA. Controls with 5 μg of anti-sense chicken TK, anti-sense β-galactosidase DNA, and anti-sense chloramphenicol acetyltransferase (CAT) DNA (Fig. 1g) all gave marginal and variable levels of either stimulation or inhibition.

As with the microinjection studies described above, the 5′ untranslated anti-sense herpes TK DNA results in a greater degree of inhibition (Table 2) of TK transformation with the herpes-chicken fusion TK gene. At a 20-fold excess of anti-sense 5′ TK to sense TK fusion gene, the inhibition was characteristically two to three times greater than that seen with herpes TK and the standard herpes anti-sense TK vector. In further parallel experiments with chicken TK and the anti-sense chicken TK expression vect r, we observed a similar 5- to 10-fold decrease in chicken TK colony f rmation compared to controls (Table 2).

We considered the possibility that the observed inhibition may have been the result of a nonspecific effect of the anti-sense expression plasmids on cell viability expression by anti-sense TK RNA. Cell cultures were transfected with sense and anti-sense herpes TK DNA under conditions where transformation would have been inhibited tenfold and each culture received in addition a "complementing" sense chicken TK plasmid. The cultures that received the additional chicken TK plasmid displayed normal transformation frequencies in HAT (hypoxanthine, aminopterin, thymidine) medium, suggesting that cells containing sense and anti-sense HSV-TK RNA are still viable (Fig. 4). As a control for nonspecific effects resulting from formation of double-stranded RNA and possible interferon production, Pestka (5) has directly assayed interferon production in our cultures that were transfected with a variety of sense and anti-sense combinations either by calcium phosphate or by DEAE-dextran methods. The very sensitive assay detected no interferon activity in these cultures.

The results with calcium phosphate–mediated TK transformation parallel the data from microinjection experiments. Anti-sense inhibition of herpes TK and chicken TK transformation is sequence specific and occurs with as little as a 10- to 20-fold excess of anti-sense DNA. Inhibition is dramatic, ranging up to 20-fold at high levels of anti-sense DNA (10- to 100-fold excess) and can be observed with as little as 52 bp of complementary 5′ untranslated RNA that does not cover the initiator AUG codon. At least two different fragments of complementary sequence (one from the protein coding region; the other from the 5′ noncoding region) can inhibit TK expression.

Transient anti-sense inhibition of the CAT gene. For transient expression assays, we utilized sense and anti-sense CAT (chloramphenicol acetyltransferase) expression plasmids (Fig. 1g). LTK⁻ cells were transfected (with the use of DEAE dextran) with a 5:1 ratio of anti-sense to sense CAT DNA; after 2 days, CAT enzyme assays were performed on cell homogenates. The anti-sense CAT plasmid caused a 5- to 20-fold decrease in activity as compared to control experiments with anti-sense herpes or chicken TK plasmids (Fig. 5). LTK⁻ cells were also stably cotransformed by calcium phosphate–mediated gene transfer with herpes TK (50 ng) as a selectable marker and including sense CAT (2 μg) and either anti-sense CAT (10 μg) or, as a control, anti-sense chicken TK (10 μg). TK-positive colonies (about 200 f r each protocol) were collected after 2 weeks, and CAT activity was measured. Under these conditions, inhibition of CAT acquence specific and ranged from 4- to 9-fold.

In a related type of analysis, an anti-sense CAT-producing cell line was constructed by cotransformation with herpes TK. The line contained about 20 copies of anti-sense CAT gene per cell and produced about 500 copies of anti sense CAT RNA. Constitutive expres sion of such an anti-sense plasmid would be predicted to provide a measure of "molecular immunity" to the subsequent expression of that gene from a sense CAT plasmid. The sense CAT plasmid DNA was introduced into the anti-sense CAT cell line and into parental LTK⁻ cells by transfection; CAT activity was assayed after transient expression. Expression of CAT activity was reduced in the anti-sense CAT cells (Fig. 5). This is not a nonspecific manifestation of inefficient DNA uptake or expression because stable transformation frequency to an APRT-positive phenotype (APRT, adenine phosphoribosyl transferase) was normal in the anti-sense CAT cell line.

These experiments extend the list of

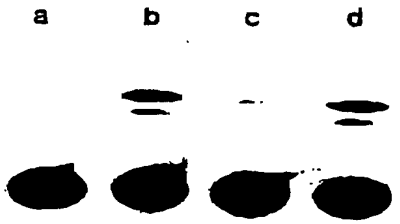

Fig. 5. Anti-sense inhibition of CAT activity. L cells were stably transformed with anti-sense CAT DNA by means of calcium phosphate–mediated cotransformation with TK. These cells each contained about 20 copies of anti-sense CAT gene and produced several hundred copies of anti-sense CAT mRNA. Then, 1 μg of sense CAT was transfected into either the anti-sense CAT polyclone line (a) or the parental LTK⁻ cells (b). CAT assays were performed after 2 days. The parental LTK⁻ cells were also transfected with 1 μg of sense CAT plus 5 μg of anti-sense CAT (c) by the DEAE-dextran procedure (*17*). Control cultures (d) received 1 μg of sense CAT and 5 μg of anti-sense herpes TK. CAT activity (*17*) was measured after 2 days.

Table 2. Inhibition of TK colony formation by cotransformation with anti-sense DNA plasmids. The transforming plasmids were used at 50 ng per plate.

*Cotransformed plasmid	Cotransformed DNA per plate (μg)	TK⁻ colonies (% control)
HSV TK		
Alone	0	100
Anti-sense ChTK†	5	123
Anti-sense β-galactosidase	5	108
pBR322	5	91
Anti-sense CAT	5	143
Anti-sense TK	1	96
	2.5	63
	5	12
HSV-chicken TK fusion		
Alone	0	100
Anti-sense 5′ TK	1	62
Anti-sense 5′ TK	2.5	17
Anti-sense 5′ TK	5	8
PBR	5	86
Chicken TK		
Alone	0	100
Anti-sense chicken TK†	5	18
pBR322	5	89
Anti-sense CAT	5	130
Anti-sense TK	5	109
Anti-sense 5′ TK	5	122

*In each experimental series there were two to ten independent transformations. Results from a typical series are shown. †Anti-sense chicken TK has chicken TK activity, probably as a result of transcription from a cryptic pBR322 promoter. This value, which is about 10 percent of the sense chicken TK activity, has been subtracted from the tabulated frequency.

genes inhibited by anti-sense expression DNA vectors to the bacterial CAT gene. suggesting that the inhibition is not limited to eukaryotic genes. In addition, the inhibition occurs during transient expression assays. and an anti-sense CAT-producing cell line is resistant r "immune" to expression from subsequently introduced CAT genes. This observation reduces the probability that the inhibition is the result of targeted recombination or conversion events that might destroy the activity of the sense CAT DNA since it is highly unlikely that homologous recombination or gene conversion could occur between the bulk of the incoming sense CAT DNA and the integrated anti-sense CAT DNA in the majority of cells in the population (*1*).

Inhibition of an endogenous herpes TK gene. Using cells previously transformed to a herpes TK-positive phenotype as a convenient model system, we have investigated whether anti-sense RNA will inhibit an endogenous gene. A collection of about 1000 TK-positive transformants was removed from HAT selection and transfected with the APRT gene and cotransforming anti-sense herpes TK DNA or as a control with APRT and cotransforming anti-sense chicken TK. APRT-positive transformants were collected (the frequency of such clones was the same for both transfections), and TK enzyme activity was measured for each culture (Fig. 6). The TK enzyme activity in cells transformed with anti-sense herpes TK was six times lower than control cultures transformed with anti-sense chicken TK. As with the CAT results described above, these data were obtained in the absence of selection for the TK gene being assayed.

To see whether the anti-sense TK transformants display any growth abnormalities in culture, the cells were grown in conventional HAT medium. Under these conditions, both anti-sense herpes TK and anti-sense chicken TK lines proliferated at comparable rates. Initially this was surprising given the fact that the TK enzyme level was significantly lower in the anti-sense TK cells. The residual level of TK activity was not zero, however, and we suspected that this reduced activity might still be sufficient for growth. If this were true, cells with lower enzyme activity might have more difficulty growing in HAT medium containing a reduced thymidine concentration. Indeed, decreasing thymidine in the medium tenfold results in growth inhibition for the anti-sense herpes TK cell line, whereas there is no detectable effect on the anti-sense chicken TK control cell lin growth even at 1/100 the normal thymidine concentration (Fig. 7).

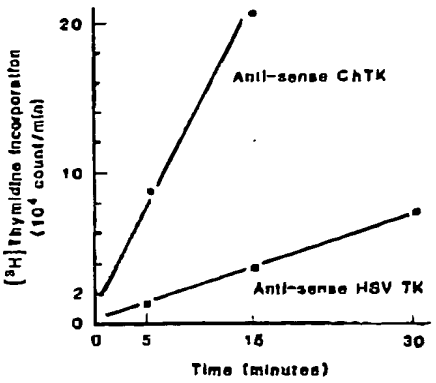

Fig. 6. Anti-sense inhibition of endogenous herpes TK activity. LTK⁻ cells were transformed to TK⁺ with HSV-TK. Cultures were then cotransformed with 5 μg (per dish) of anti-sense herpes TK or anti-sense chicken TK (ChTK) and an APRT plasmid. APRT-positive cells were selected, and TK enzyme activity was determined (*18*).

Conditional expression of anti-sense TK RNA. As with any mutational analysis, it will be difficult to discern the function of a crucial gene by anti-sense inhibition when the suppression of that gene has lethal consequences. One way to deal with this problem is to make the expression of the anti-sense RNA conditional. for example, by using an inducible promoter. In order to test whether conditional anti-sense inhibition is possible, we used the dexamethasone-inducible MMTV LTR to drive the synthesis of anti-sense herpes TK (Fig. 1h). Collections of about 500 herpes TK-p sitive colonies were first selected in HAT medium after calcium phosphate–mediated DNA transformation of TK⁻, APRT⁻ L cells with wild-type HSV-TK gene. Cultures were then divided in tw parts, and one was cotransformed with APRT plus a control anti-sense chicken TK plasmid and the second was cotransformed with APRT and the MMTV anti-sense HSV-TK plasmid. The frequency of APRT-positive colonies was similar for the two cultures, and polyclones (about 50) from each culture were grown. Since the basal and induced levels of activity f the MMTV promoter vary from clone to clone (*6*), the polyclones were taken through two subsequent selections in HAT medium to ensure that the resident sense TK gene was still functioning efficiently. Cells with a high basal level of inhibitory anti-sense TK would also be selected against because they would presumably have lower TK activity. Then the cells were again passaged in APRT selection medium to ensure that they had retained the APRT gene and the cotransfected anti-sense TK gene. Finally, the cells were grown in varying concentrations of dexamethasone for 4 to 8 days (to allow for enzyme turnover), and then TK enzyme levels were determined (Fig. 8). Control cultures containing the anti-sense chicken TK gene had TK enzyme levels that were usually unaffected by dexamethasone. In contrast, after treatment with 10^{-6} or $10^{-7}M$ dexamethasone, a dose-dependent decrease (five-

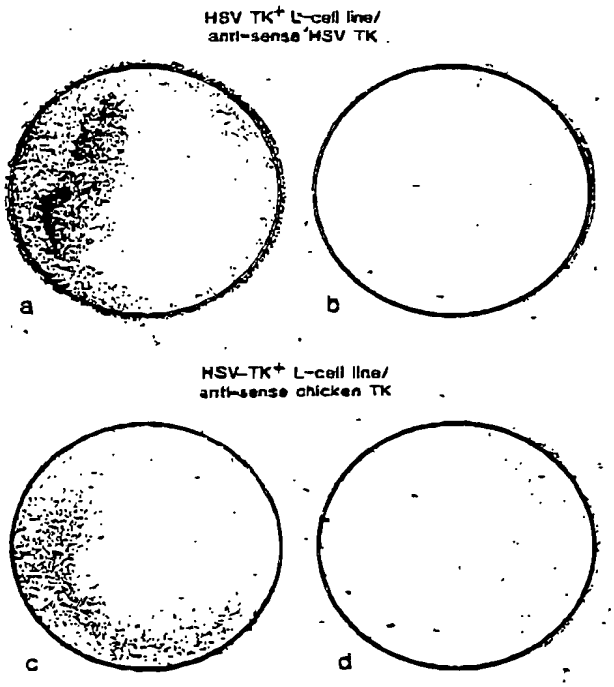

Fig. 7. Anti-sense inhibition of endogenous herpes TK phenotype. The anti-sense HSV-TK (a and b) or the anti-sense chicken TK (c and d) lines described in Fig. 6 were grown in normal HAT medium ($1.5 \times 10^{-5}M$ thymidine) or in HAT medium with one-tenth ($10^{-6}M$) or one hundredth ($10^{-7}M$) the usual amount of thymidine and stained after 1 week in culture. Both cell lines proliferated in normal HAT medium (a and c). The anti-sense HSV-TK line was unable to grow in HAT medium with one-tenth the normal concentrations of thymidine (b), whereas anti-sense chicken TK line grew well at one-hundredth the normal level of thymidine (d).

ly found. While normal TK$^+$ cells grew almost as well in the presence of or absence of these concentrations of dexamethasone, cells that also contained the inducible anti-sense TK gene grew more slowly in the presence of $10^{-7}M$ dexamethasone (Fig. 8, inset) when put under HAT selection. When put under APRT selection, however, these cells grew normally in the presence of these concentrations of dexamethasone. This model system suggests that dexamethasone can be used to provide anti-sense transcription that can be regulated and, as a consequence, conditional inhibition of gene activity. Recent work suggests that a heatshock promoter can also be used to produce a conditional anti-sense phenotype (*7*).

Inhibition of endogenous actin gene expression. To test whether a normal cellular gene could be inhibited by anti-sense RNA, we constructed an anti-sense DNA expression vector that would produce anti-sense cytoplasmic β-actin (Fig. 1f). We anticipated that cotransformation of TK and anti-sense actin DNA that have taken up both genes because the phenotype of actin mutations in yeast is cell lethality (*8*). This can be assayed by monitoring the frequency of TK-positive transformed colonies. The results show that an anti-sense actin DNA plasmid dramatically decreases the frequency of normal TK-positive colonies as compared to parallel control cotransformations with insertless plasmids and anti-sense chicken TK plasmids (Fig. 9). Interestingly, the frequency of small colonies (four to ten cells) during the first several days after transfection was similar in control and anti-sense actin-transformed cultures. Subsequently, cell proliferation diminished in the cultures transformed with anti-sense actin. This probably indicates that the initial transformation of these cells is normal, but that the anti-sense actin DNA causes a sufficient decrease in actin production so that the cells can sustain normal growth for only two to three divisions.

To test whether cells containing anti-sense actin RNA show a structural phenotype, BSC-1 cells were injected with the anti-sense actin DNA, and 4 days later the cells were stained with *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin (*9*) to visualize actin cables. The cells were counterstained with propidium iodide, to stain the nuclear DNA and the perinuclear RNA. While BSC-1 cells display typically dramatic actin microfilament bundles after injection with anti-sense TK DNA, only about half of the cells show normal actin cables after injection of anti-sense actin DNA. This corresponds to the fraction of cells expected to express anti-sense actin RNA efficiently after microinjection. As was observed in the transfection experiments, cells with anti-sense actin plasmid show no obvious change in cell morphology in the first several days after microinjection, and the distribution of cytoplasmic RNA as assayed by propidium staining is unaltered. In contrast, the quantity and length of actin microfilament bundles in the affected cells was markedly reduced although the actin staining along the cell periphery, especially in the areas of active ruffling, appeared to be undiminished (overexposed in Fig. 10 to emphasize the diminution of the stress fiber array). This suggests that when actin levels are decreased the peripheral actin filaments are preferentially retained.

The decrease in actin cables is also observed 24 to 36 hours after capped anti-sense actin RNA is microinjected directly into BSC-1 cells (Fig. 10, b and c). Microinjection of anti-sense TK RNA has no obvious effect on the actin microfilament distribution. This RNA was made in vitro by means of an SP6 promoter ligated to anti-sense actin DNA encoding a 500-bp fragment of 5′ actin cDNA. Introduction of anti-sense actin RNA results in the disruption of actin cables, presumably because of a lower actin monomer concentration in injected cells.

Implications of anti-sense gene inhibition. The cytoplasmic manifestations of anti-sense actin inhibition and the subsequent inhibition of cell proliferation are striking. This analysis has yielded the interesting and unanticipated observation that actin microfilament cables are more labile to changes in actin monomer dynamics than the actin filaments present in the cell periphery. While we cannot readily assess the extent of actin synthesis inhibition in microinjected cells, the observed phenotypic changes suggest that any feedback mechanisms that may serve to regulate actin pool size are unable to compensate for the anti-sense actin perturbation.

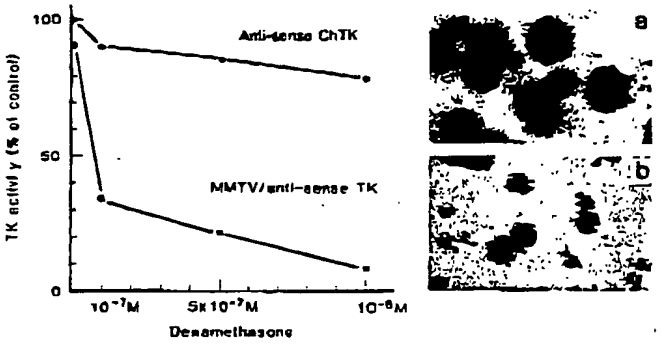

Fig. 8. Dexamethasone induction of MMTV anti-sense TK. Herpes TK-positive L cells were cotransformed with the inducible MMTV anti-sense TK plasmid (Fig. 1h) under APRT selection; control cultures were cotransformed with anti-sense chicken TK plasmid DNA. Transformed colonies were grown up and taken through successive rounds of HAT selection followed by APRT selection. Control and anti-sense TK cultures were then induced with increasing levels of dexamethasone, and TK enzyme activity was assayed after 5 days. The insets show representative colonies (a) from a control culture and (b) from a dexamethasone-treated culture, illustrating the difference in growth induced by dexamethasone in the MMTV anti-sense TK-containing cells. These differences are most dramatic at $10^{-7}M$ dexamethasone and do not occur in the absence of HAT selection. However, at slightly higher concentrations of dexamethasone ($5 \times 10^{-7}M$) an inhibition of cell growth is observed that is independent of HAT selection. [×13]

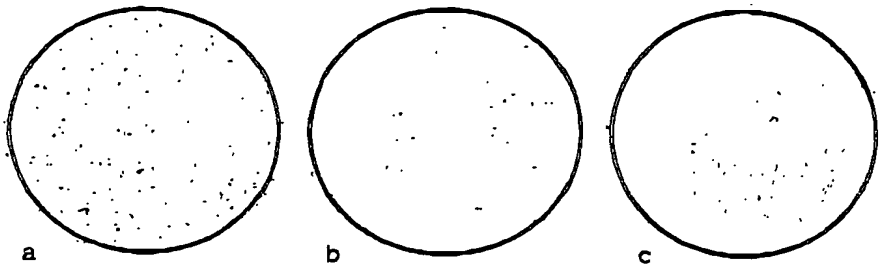

Fig. 9. Inhibition of TK colony formation by anti-sense actin plasmid DNA. LTK$^-$ cells were cotransformed with herpes TK DNA (50 ng per dish), with calf thymus (carrier) DNA (10 μg per dish) and with (a) control anti-sense chicken DNA or (b) 1 μg or (c) 10 μg of anti-sense actin DNA. Colonies were stained after 2 weeks selection in HAT medium.

This study extends the number of genes whose expression has successfully been suppressed by anti-sense RNA and suggests that the technique may provide a general methodology for genetic analysis. In addition to the original work with herpes TK, we have shown that chicken TK, CAT, and cytoplasmic actin can be suppressed and others have shown that β-globin (*10*), β-galactosidase (*11*), the *Drosophila* krüppel gene (*12*), and a *Xenopus* gap junction gene (*13*) can be inhibited by anti-sense RNA. The cell types that have been used in these experiments include L cells, 3T3 cells, *Xenopus* oocytes, *Drosophila* embryos, and frog embryos. The method of delivery ranges from microinjection to calcium phosphate–mediated stable transformation, to DEAE–dextran mediated transient expression; the reagents include "flipped" plasmid DNA vectors designed to produce anti-sense RNA, synthetic anti-sense RNA, and synthetic oligonucleotides. While most work has been done inhibiting experimentally introduced genes, several endogenous genes (integrated herpes TK, actin, krüppel, and gap junction) have also been inhibited. The inhibition can be accomplished efficiently with as little as 52 bp of homology to the 5′ untranslated region of the sense mRNA and does not have to include the initiator AUG. When the anti-sense vector is linked to the dexamethasone-inducible MMTV promoter, dexamethasone-inducible inhibition of TK activity is observed. Conditional anti-sense inhibition has also been achieved with heatshock gene promoter sequences (*7*).

Many questions remain with regard to the mechanism of inhibition, and some of the available data is fragmentary. In addition to the anti-sense actin experiment (Fig. 10), anti-sense inhibition by anti-sense RNA synthesized in vitro has been demonstrated in frog oocytes by separately microinjecting sense and anti-sense RNA's for *Xenopus* β-globin (*10*),

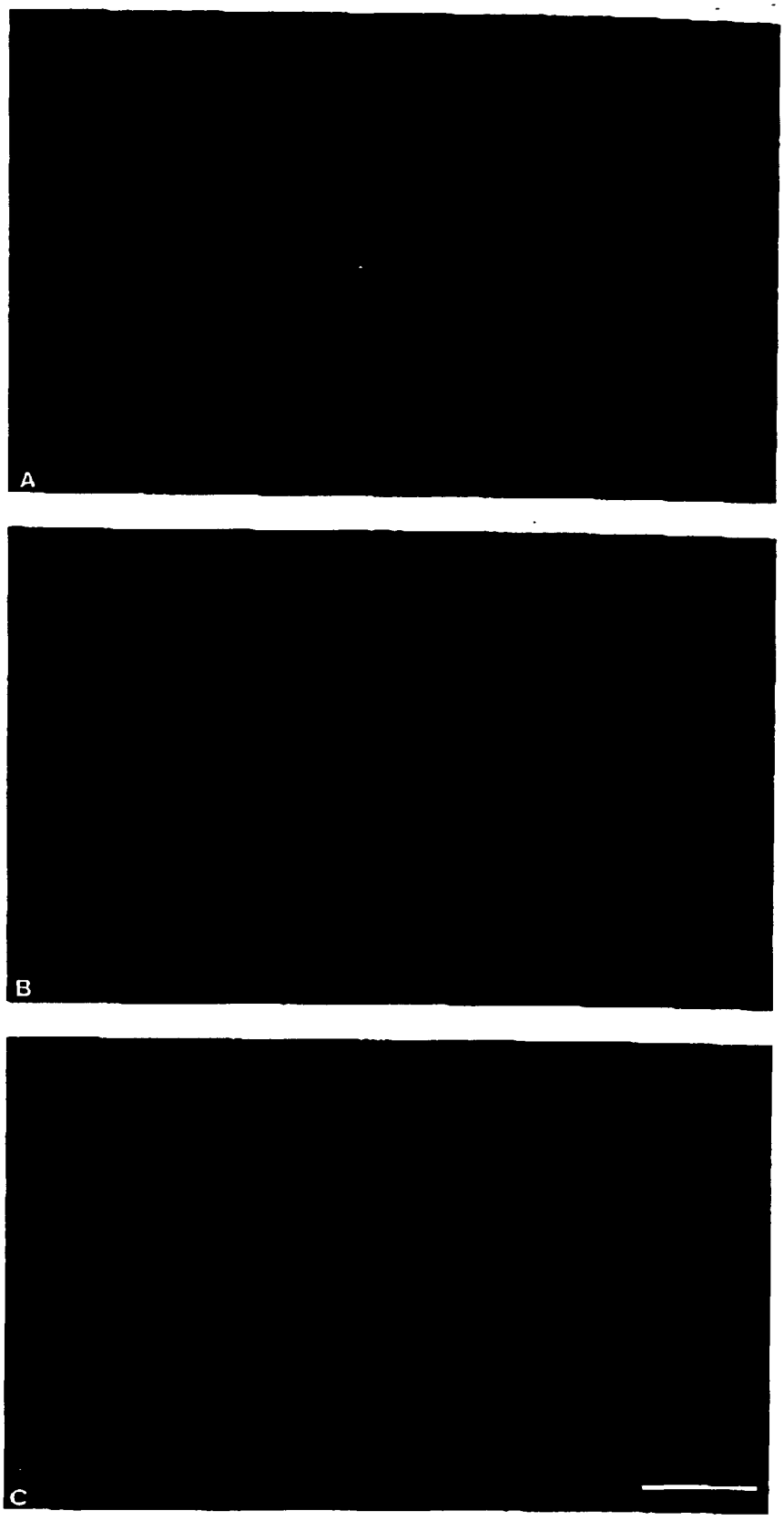

Fig. 10. Inhibition of actin cable formation by anti-sense actin RNA. Capped RNA was synthesized in vitro with SP6 RNA polymerase and plasmids that direct the synthesis of either (a) sense or (b and c) anti-sense actin RNA. In vitro capping was achieved by including 5 m*M* diguanosine triphosphate $G(5')p_3(5')G$ in the reaction (*14*). 0.1 pl of RNA at 0.5 μg/μl was injected into BSC-1 cells and after 30 hours the cells were fixed and stained with fluorescent phalloidin (green) (*9*) to stain filamentous actin and propidium iodide (red-orange) to stain DNA and RNA. Similar results are also obtained with the anti-sense actin plasmid DNA shown in Fig. 1f.

for herpes TK (*14*), and for CAT (*14*). This is an attractive system for studying the mechanics of anti-sense inhibition since it is possible to monitor the levels and integrity of sense and anti-sense transcripts during the course of an experiment. By means of this approach, double-stranded RNA hybrid molecules have been detected in oocytes as the putative intermediate in the inhibition process (*10*). Moreover, *Xenopus* β-globin mRNA's already loaded onto oocyte polysomes are susceptible to anti-sense inhibition, indicating that inhibition can be cytoplasmic and thus probably translational. These results, however, do not exclude the possibility of a nuclear anti-sense inhibition process as well. Indeed, Kim and Wold have shown that anti-sense TK RNA forms duplexes with sense TK RNA, and that the duplexes are preferentially localized in the cell nucleus (*15*).

How stable are anti-sense RNA's? In L cells anti-sense TK RNA may be less stable than sense RNA (*1*), whereas in frog oocytes, both are extremely stable (*14*). Sense and anti-sense CAT gene transcripts are stable in cultured cells as well as in oocytes (*14*). Can the inhibition b described by simple solution-type hybridization kinetics and how much of an excess of anti-sense RNA is usually necessary? In frog oocytes the kinetics of inhibition are generally consistent with hybridization theory predictions (*14*); however, the various amounts of anti-sense RNA needed to achieve significant inhibition in different systems is puzzling. F r example, in *Xenopus* oocytes, a 5- to 50-fold excess of anti-sense over sense RNA results in a 10- to 20-fold inhibition of gene activity. When cultured s matic cells are used, a 1:1 ratio of sense to anti-sense β-galactosidase DNA yi lded a 95 percent inhibition of activity (*11*); we found that a minimum rati of 5:1 anti-sense to sense DNA is required for herpes TK inhibition in transfection studies and a ratio of 50:1 is required in microinjection studies to achieve comparable levels of inhibition.

We do not know how secondary structure and ribonucleoprotein (RNP) packaging of both the sense and anti-sense RNA's affect inhibition nor do we have unambiguous information on which regions of an RNA transcript are most susceptible to anti-sense inhibition. In frog oocytes β-globin is not inhibited by a 3′ anti-sense RNA (*10*), but CAT mRNA is (*14*), although in both cases the 5′-anti-sense probe is more effective. Our results also suggest that 5′ anti-sense may be a more efficient inhibitor in cultured cells than protein coding sequences alone. Further, this may explain why naturally occurring prokaryotic anti-sense paradigms utilize anti-sense transcripts complementary to the 5′ regions of their respective target transcripts (*2*, *3*).

It will actually be interesting to determine if anti-sense transcription functions as a normal cellular control mechanism in higher eukaryotes as it does in prokaryotes.

The observations on anti-sense 5′ TK also present a potential strategy for experimental gene switching manipulations and even gene therapy protocols wherein the gene of interest, either a normal or a deleterious gene, is inhibited by anti-sense RNA complementary to a nontranslated (possibly even an intronic) domain of the RNA transcript. A sense orientation RNA or DNA coding for the desired gene product could be introduced concomitantly; however, this gene would be designed so that it is missing the nontranslated sequences complementary to the anti-sense inhibitor. In this fashion a gene could be effectively "replaced" by a substitute gene that differs by as little as one base pair without having to alter the genomic copy of that gene.

Finally, it is important to know the general applicability of the anti-sense phenomenon and the efficiency of the available mechanical and viral methods for introducing anti-sense DNA and RNA into cells if this procedure is to be used as an alternative to genetic analysis in higher organisms. Our own experience promotes optimism although the number of examples is still limited and the details of the molecular mechanism of anti-sense inhibition are not known. A better understanding of the various facets of anti-sense inhibition will contribute to studies with the use of cell type–specific anti-sense RNA's to identify and characterize regulatory genes that are involved in cellular morphogenesis and embryogenesis.

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STEDMAN'S

Medical Dictionary

26th Edition

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spherical a., a monochromatic a. occurring in refraction at a spherical surface in which the paraxial and peripheral rays focus along the axis at different points. SYN dioptric a.

ventricular a., SYN aberrant ventricular *conduction*.

ab·er·rom·e·ter (ab-er-rom'ĕ-ter). An instrument for measuring optical aberration or any error in experimentation. [L. *aberratio*, aberration, + G. *metron*, measure]

abe·ta·lip·o·pro·tein·e·mia (ā-bā'tă-lip'ō-prō'tēn-ē'mē-ă) [MIM*200100]. A disorder characterized by an absence from plasma of low density lipoproteins that migrate electrophoretically as beta globules, presence of acanthocytes in blood, retinal pigmentary degeneration, malabsorption, engorgement of upper intestinal absorptive cells with dietary triglycerides, and neuromuscular abnormalities; autosomal recessive inheritance. SYN Bassen-Kornzweig syndrome. [G. *a-*, priv., + β, + lipoprotein + *-emia*, blood]

normotriglyceridemic a., a. with normal levels of triglycerides. This inherited disorder (possibly autosomal recessive) is probably due to the absence of apolipoprotein B-100.

abey·ance (ă-bā'ans). A state of temporary abolition of function. [fr. O. Fr.]

ab·far·ad (ab-far'ad). Electromagnetic unit of capacity equal to 10^9 farads.

ABG Abbreviation for arterial blood gas. SEE blood *gases*, under *gas*.

ab·hen·ry (ab-hen'rē). Electromagnetic unit of inductance equal to 10^{-9} henry.

ab·i·ent (ab'ē-ent). Having a tendency to move away from the source of a stimulus, as opposed to adient. [L. *abiens*, fr. *ab- eo*, to go from]

abil·i·ty (ă-bil'i-tē). The physical, mental, or legal competence to function. [L. *habilitas*, aptitude]

abi·ot·ic (ā-b-ī-ot'ik). **1.** Incompatible with life. **2.** Without life.

ab·i·ot·ro·phy (ab-ē-ot'rō-fē). An age-dependent manifestation of a trait that being genetically determined has been latent from the time of conception. [G. *a-* priv. + *bios*, life, + *trophē*, nourishment]

ab·ir·ri·tant (ab-ir'i-tănt). **1.** Obsolete term for soothing, or relieving irritation. **2.** Obsolete term for an agent possessing this property.

ab·ir·ri·ta·tion (ab-ir-i-tā'shŭn). Obsolete term for diminution or abolition of irritability in a part. [L. *ab*, from, + *irrito*, pp. *-atus*, to irritate]

ab·ir·ri·ta·tive (ab-ir'i-tā-tiv). Obsolete term for abirritant.

abl. An oncogene found in the Abelson strain of mouse leukemia virus and involved in the Philadelphia chromosome translocation in chronic granulocytic leukemia.

ab·lac·ta·tion (ab-lak-tā'shŭn). SYN weaning (1). [L. *ab*, from, + lactation]

ablas·te·mic (ā-blas-tem'ik). Not germinal or blastemic. [G. *a-* priv. + *blastēma*, sprout]

ablas·tin (ă-blas'tin). An antibody that seems to inhibit reproduction of trypanosomes; found in rats infected with *Trypanosoma lewisi*. [G. *a-* priv. + *blastos*, germ]

ab·late (ab-lāt'). To remove, or to destroy the function of. [L. *auferō*, pp. *ab- latus*, to take away]

ab·la·tion (ab-lā'shun). Removal of a body part or the destruction of its function, as by a surgical procedure, morbid process, or noxious substance. [L. see ablate]

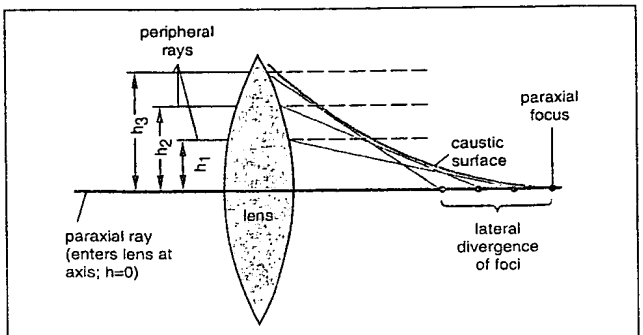
electrode catheter a., a method of ablating the site of origin of arrhythmias whereby high energy elective shocks are delivered by intravascular catheters.

ab·la·tio pla·cen·tae (ab-lā'shē-ō pla-sen'tē). SYN abruptio placentae.

ableph·a·ria (ā-blef-ar'ē-ă) [MIM*200110]. Congenital absence, partial or complete, of the eyelids; recessive inheritance. SEE ALSO cryptophthalmus. [G. *a-* priv. + *blepharon*, eyelid]

ab·lu·ent (ab'lū-ent). **1.** Cleansing. **2.** Anything with cleansing properties. [L. *abluens*, fr. *ab-luo*, to wash off]

ab·lu·tion (ab-lū'shŭn). An act of washing or bathing. [L. *ablutio*, washing off, cleansing]



aberration (spherical)

ab·lu·to·ma·nia (ab-lū-tō-mā'nē-ă). Rarely used term for a morbid preoccupation with thoughts about cleanliness, exhibited by frequent washing, as seen in obsessive-compulsive disorder. [L. *ablutio*, washing, + G. *mania*, insanity]

ab·ner·val (ab-ner'văl). Away from a nerve; denoting specifically a current of electricity passing through a muscular fiber in a direction away from the point of entrance of the nerve fiber. SYN abneural (1).

ab·neu·ral (ab-nūr'ăl). **1.** SYN abnerval. **2.** Away from the neural axis. [L. *ab*, away from, + G. *neuron*, nerve]

ab·nor·mal (ab-nōr'măl). Not normal; differing in any way from the usual state, structure, condition, or rule.

ab·nor·mal·i·ty (ab-nōr-mal'i-tē). **1.** The state or quality of being abnormal. **2.** An anomaly, deformity, malformation, impairment, or dysfunction.

figure-of-8 a., a radiographic appearance associated with total anomalous drainage of the pulmonary venous circulation into enlarged right and anomalous left venae cavae, that produces a globular density above the heart; the silhouette suggests the figure 8. SEE snowman a. SYN snowman a.

snowman a., SYN figure-of-8 a.

ABO blood group. See Blood Groups appendix.

ABO blood group

blood group	genotype	frequency in U.S. (%)	antigens of erythrocytes	antibodies in serum
A	AA or AO	32 pos 7 neg	A	anti-B (β)
B	BB or BO	9 pos 2 neg	B	anti-A (α)
AB	AB	3 pos 1 neg	A and B	none
O	OO	38 pos 8 neg	neither A nor B	α and β

ab·ohm (ab'ōm). Electromagnetic unit of resistance equal to 10^{-9} ohm.

aboi·e·ment (ah-bwah-mahn'). Rarely used term for the involuntary production of abnormal sounds, as seen in Gilles de la Tourette syndrome. [Fr. barking, yelping]

ab·o·ma·si·tis (ab'ō-mas-ī'tis). Inflammation of the abomasum.

ab·o·ma·sum (ab-ō-mā'sŭm). The fourth compartment and the glandular portion of the stomach of a ruminant. [L. *ab*, from, + *omasum*, bullock's tripe]

ab·o·rad, ab·o·ral (ab-ō'rad, -răl). In a direction away from the mouth; opposite of orad. [L. *ab*, from, + *os* (*or-*), mouth]

ventral tegmental d., SEE tegmental d.'s (2).

Wernekinck's d., SYN d. of superior cerebellar peduncles.

de·cus·sa·ti·o·nes (dē-kŭs-ā-shē-ō'nēz). Plural of decussatio.

de·den·ti·tion (dē-den-tish'ŭn). Obsolete term denoting loss of teeth.

de·dif·fer·en·ti·a·tion (dē-dif'er-en-shē-ā'shŭn). 1. The return of parts to a more homogeneous state. 2. SYN anaplasia.

de·do·la·tion (dē-dō-lā'shŭn). A slicing wound made by a sharp instrument grazing the surface. [L. *de-dolo,* pp. *-atus,* to hew away]

de·duc·tion (dē-duk'shun). The logical derivation of a conclusion from certain premises. The conclusion will be true if the premises are true and the deductive argument is valid. Cf. induction (9).

de·ef·fer·en·ta·tion (dē-ef-er-en-tā'shŭn). A loss of the motor nerve fibers to an area of the body. [L. *de,* from, + efferent]

deep (dēp). SYN profundus.

de·ep·i·car·di·al·i·za·tion (dē-ep-i-kar'dē-al-i-zā'shŭn). Surgical destruction of the epicardium, usually by the application of phenol, designed to promote collateral circulation to the myocardium.

Deetjen, Hermann, German physician, 1867–1915. SEE D.'s *bodies,* under *body.*

def, DEF Abbreviation for decayed, extracted, and filled tooth. SEE def caries *index.*

de·fat·i·ga·tion (dē-fat-i-gā'shŭn). Weariness, exhaustion, or extreme fatigue. [L. *de-fatigo,* pp. *-atus,* to tire out]

def·e·cate (def'ĕ-kāt). To perform defecation.

def·e·ca·tion (def-ĕ-kā'shŭn). The discharge of feces from the rectum. SYN motion (2), movement (3). [L. *defaeco,* pp. *-atus,* to remove the dregs, purify]

de·fec·og·ra·phy (de-fē-kog'ră-fē). Radiographic examination of the act of defecation of a radiopaque stool. [defecation + G. *graphō,* to write]

de·fect (dē'fekt). An imperfection, malformation, dysfunction, or absence; an attribute of quality, in contrast with deficiency, which is an attribute of quantity. [L. *deficio,* pp. *-fectus,* to fail, to lack]

aortic septal d., aorticopulmonary septal d., a small congenital opening between the aorta and pulmonary artery about 1 cm above the semilunar valves, *e.g.,* aorticopulmonary window. SYN aorticopulmonary window.

atrial septal d., a congenital d. in the septum between the atria of the heart, due to failure of the foramen primum or secundum to close normally; may involve atrioventricular canal cushions; occasionally there is strong evidence of autosomal dominant inheritance [MIM*108800]. In varying degree, it is also a common feature of the autosomal recessive Ellis-van Creveld *syndrome* [MIM*225500] and the autosomal dominant Holt-Oram *syndrome* [MIM*142900].

atrial ventricular canal d., a d. caused by deficient or absent septal tissue immediately above and below the normal level of the atrioventricular valves, including the region normally occupied by the A-V septum in hearts with two ventricles. The A-V valves are abnormal to a varying degree.

birth d., d. present at birth; sometimes referred to as congenital d.

congenital ectodermal d., incomplete development of the epidermis and skin appendages; the skin is smooth and hairless, the facies abnormal, and the teeth and nails may be affected; sweating may be deficient. SYN congenital ectodermal dysplasia.

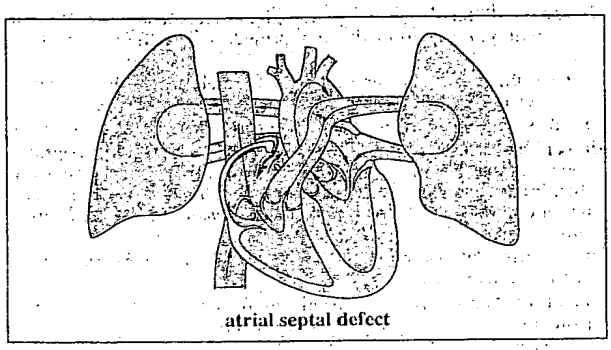
coupling d., SEE familial *goiter.*

Eisenmenger's d., SYN Eisenmenger's *complex.*

endocardial cushion d., SYN persistent atrioventricular *canal.*

fibrous cortical d., a common 1 to 3 cm d. in the cortex of a bone, most commonly the lower femoral shaft of a child, filled with fibrous tissue. Nonosteogenic or nonossifying *fibroma* by convention refers to lesions greater than 3 cm in diameter. SEE ALSO nonossifying *fibroma.* SYN nonosteogenic fibroma.

filling d., displacement of contrast medium by a space-occupying lesion in a radiographic study of a contrast-filled hollow viscus, such as a polyp on a barium enema; also applied to defects in the otherwise uniform distribution of radionuclide in an organ, such as a metastasis in the liver on a ^{99m}Tc-sulfur colloid scan.



atrial septal defect

Gerbode d., a defect in the interventricular portion of the membranous septum, associated with a communication between the right ventricle and the right atrium through an abnormality in the tricuspid valve.

iodide transport d., SEE familial *goiter.*

iodotyrosine deiodinase d., SEE familial *goiter.*

luteal phase d., a condition characterized by inadequate secretion of progesterone during the luteal phase of the menstrual cycle, with resultant sterility; subnormal luteal function commonly attributed to abnormal pituitary gonadotropin secretion. SYN luteal phase deficiency.

metaphysial fibrous cortical d., a small (less than 2 to 3 cm in diameter) fibrous cortical d.

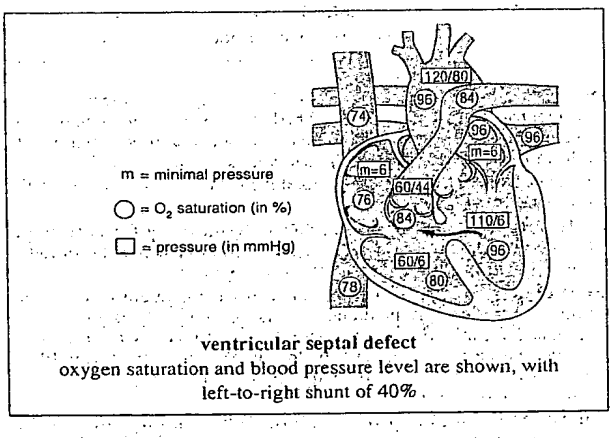
organification d., SEE familial *goiter.*

osteoporotic marrow d. (ōs'tē-ō-pō-ro'tik), focal osteoporotic bone marrow d. of the jaw; a focal radiolucent d. composed of normal marrow.

postinfarction ventricular septal d., a d. developed in the ventricular septum resulting from rupture of an acute myocardial infarction.

salt-losing d., renal tubular abnormality causing loss of sodium in the urine.

ventricular septal d., a congenital d. in the septum (membranous or muscular) between the cardiac ventricles, usually resulting from failure of the spiral septum to close the interventricular foramen.



ventricular septal defect
oxygen saturation and blood pressure level are shown, with left-to-right shunt of 40%.

de·fec·tive (dē-fek'tiv). Denoting or exhibiting a defect; imperfect; a failure of quality.

de·fem·i·na·tion (dē-fem-i-nā'shŭn). A weakening or loss of feminine characteristics. [L. *de-,* away, + *femina,* woman]

SYN physaliferous. [G. *physallis*, bladder, bubble, + *phoros*, bearing]

phys·a·lis (fis'ă-lis). A vacuole in a giant cell found in certain malignant neoplasms, such as chordoma. [G. *physallis*, a bladder]

Phy·sa·lop·tera (fī'să-lop'ter-ă, fis-). A large genus of spiruroid roundworms parasitic in the stomach and duodenum of vertebrates, especially birds and mammals; they are transmitted via insect and annelid intermediate hosts and are frequently pathogenic, causing erosions and catarrhal gastritis. *P. caucasica* is a species reported in man in the southern part of the area formerly known as the USSR; *P. mordens* is a species from tropical Africa found only rarely in the esophagus, stomach, and intestine of man (probably cases of temporary infection from ingestion of infected insects). [G. *physallis*, bladder, + *pteron*, wing]

phy·sa·lop·ter·i·a·sis (fī'să-lop-ter-ī'ă-sis). Infection of animals and man with nematodes of the genus *Physaloptera*.

phys·e·al (fiz'ē-ăl). Pertaining to the physis, or growth cartilage area, separating the metaphysis and the epiphysis.

△**physi-.** SEE physio-.

phys·i·at·ric·i·an (fix'ē-ă-trish'ŭn). A physician who specializes in physiatry (rehabilitation medicine).

phys·i·at·rics (fiz-ē-at'riks). **1.** Old term for physical *therapy*. **2.** Rehabilitation management. [G. *physis*, nature, + *iatrikos*, healing]

phys·i·a·trist (fiz-ī'ă-trist). A physician who specializes in physical medicine.

phys·i·a·try (fi-zī'ă-trē). SYN physical *medicine*.

phys·ic (fiz'ik). **1.** The art of medicine. **2.** A medicine, often a lay term for a cathartic. [G. *physikos*, natural, physical]

phys·i·cal (fiz'i-kăl). Relating to the body, as distinguished from the mind. [Mod. L. *physicalis*, fr. G. *physikos*]

phy·si·cian (fi-zish'ŭn). **1.** A doctor; a person who has been educated, trained, and licensed to practice the art and science of medicine. **2.** A practitioner of medicine, as contrasted with a surgeon. [Fr. *physicien*, a natural philosopher]

attending p., (1) p. responsible for the care of a patient; (2) p. supervising the care of patients by interns, residents, and/or medical students. (3) a doctor who has completed internship and residency.

family p., a p. who specializes in family practice.

osteopathic p., a practitioner of osteopathy. SYN osteopath.

resident p., SYN resident.

phy·si·cian as·sis·tant (P.A.). A person who is trained, certified, and licensed to perform history taking, physical examination, diagnosis, and treatment of commonly encountered medical problems, and certain technical skills, under the supervision of a licensed physician, and who thereby extends the physician's capacity to provide medical care. Many subspecialties exist, such as orthopedist's assistant, sports injury assistant, pediatrician's assistant, etc.

Physick, Philip Syng, U.S. surgeon, 1768–1837: SEE P.'s *pouches*, under *pouch*.

phys·i·co·chem·i·cal (fiz'i-kō-kem'i-kăl). Relating to the field of physical chemistry.

phys·ics (fiz'iks). The branch of science concerned with the phenomena of matter and energy and their interactions. SEE physic.

radiation p., the scientific discipline of the application of p. to the use of ionizing radiation in therapy and in diagnostic radiology; including, by extension, nuclear medicine applications, ultrasound, and magnetic resonance imaging.

△**physio-, physi-.** Physical, physiological; 2. Natural, relating to physics. [G. *physis*, nature]

phys·i·o·gen·ic (fiz'ē-ō-jen'ik). Related to or caused by physiologic activity. [physio- + G. *genesis*, origin]

phys·i·og·no·my (fiz-ē-og'nō-mē). **1.** The physical appearance of one's face, countenance, or habitus, especially regarded as an indication of character. **2.** Estimation of one's character and mental qualities by a study of the face and other external bodily features. [physio- + G. *gnōmōn*, a judge]

phys·i·og·no·sis (fiz-ē-og-nō'sis). Diagnosis of disease based upon a study of the facial appearance or bodily habitus. [physio- + G. *gnōsis*, knowledge]

phys·i·o·log·ic, phys·i·o·log·i·cal (fiz-ē-ō-loj'ik, -loj'i-kăl). **1.** Relating to physiology. **2.** Normal, as opposed to pathologic; denoting the various vital processes. **3.** Denoting something that is apparent from its functional effects rather than from its anatomical structure; *e.g.*, a p. sphincter. **4.** Denoting a dose or the effects of such a dose (of a chemical agent that either is or mimics a hormone, neurotransmitter, or other naturally occurring agent) that is within the range of concentrations or potencies that would occur naturally. Cf. homeopathic (2), pharmacologic (2), supraphysiologic.

phys·i·o·log·i·co·an·a·tom·i·cal (fiz'ē-ō-loj'i-kō-an-ă-tom'i-kăl). Relating to both physiology and anatomy.

phys·i·ol·o·gist (fiz-ē-ol'ō-jist). A specialist in physiology.

phys·i·ol·o·gy (fiz-ē-ol'ō-jē). The science concerned with the normal vital processes of animal and vegetable organisms, especially as to how things normally function in the living organism rather than to their anatomical structure, their biochemical composition, or how they are affected by drugs or disease. [L. or G. *physiologia*, fr. G. *physis*, nature, + *logos*, study]

comparative p., the science concerned with the differences in the vital processes in different species of organisms, particularly with a view to the adaptation of the processes to the specific needs of the species, to illuminating the evolutionary relationships among different species, or to establishing other interspecific generalizations and relationships.

general p., the science of the functions or vital processes common to almost all living things, whether animal or plant, as opposed to aspects of p. peculiar to particular types of animals or plants, or to the application of p. to applied sciences such as medicine and agriculture.

hominal p., p. as applied to the elucidation of the normal functions of the human being.

pathologic p., that part of the science of disease concerned with disordered function, as distinguished from anatomical lesions. SYN physiopathology.

phys·i·o·med·i·cal (fiz-ē-ō-med'i-kăl). Denoting the use of physical rather than medicinal measures in the treatment of disease.

phys·i·o·path·o·log·ic (fiz'ē-ō-path-ō-loj'ik). Relating to pathologic physiology.

phys·i·o·pa·thol·o·gy (fiz'ē-ō-pă-thol'ō-jē). SYN pathologic *physiology*.

phys·i·o·psy·chic (fiz'ē-ō-sī'kik). Pertaining to both mind and body.

phys·i·o·py·rex·ia (fiz'ē-ō-pī-rek'sē-ă). Fever produced by a physical agent. [physio- + G. *pyrexis*, feverishness]

phys·i·o·ther·a·peu·tic (fiz'ē-ō-thār-ă-pyū'tik). Pertaining to physical *therapy*.

phys·i·o·ther·a·pist (fiz'ē-ō-thār'ă-pist). A physical therapist. SEE physical *therapy* (2).

phys·i·o·ther·a·py (fiz'ē-ō-thār'ă-pē). SYN physical *therapy* (1). [physio- + G. *therapeia*, treatment]

oral p., the use of a toothbrush, interdental stimulator, floss, irrigating device, or other adjunctive aid to maintain oral health.

phy·sique (fi-zēk'). constitutional type; the physical or bodily structure; the "build." [Fr.]

phy·sis (fī'sis). A term sometimes used in referring to the epiphysial cartilage. [G. growth]

△**physo-.** **1.** Tendency to swell or inflate. **2.** Relation to air or gas. [G. *physaō*, to inflate, distend]

phy·so·cele (fī'sō-sēl). **1.** A circumscribed swelling due to the presence of gas. **2.** A hernial sac distended with gas. [physo- + G. *kēlē*, tumor, hernia]

Phy·so·ceph·a·lus sex·a·la·tus (fī'sō-sef'ă-lŭs sek'să-lā'tŭs). A small species of spiruroid nematodes (family Spiruridae) found in the stomach of pigs, horses, camels, rabbits, and hares; worldwide in distribution, and especially prevalent in hogs. [G. *physa*, bellows, + *kephalē*, head]

phy·so·ceph·a·ly (fī-sō-sef'ă-lē). Swelling of the head resulting

DNA TUMOR VIRUSES

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Similarity between SV40 and Polyoma Virus

Almost from the moment of its discovery, SV40 has been intensively studied. Cell culture systems for studying either lytic replication of the virus or transformation were quickly devised (Todaro and Green 1964; Black 1966), as were procedures for purifying the viral particles (Black et al. 1964), and it soon became obvious that SV40 is very similar to polyoma virus. The two viruses have identical structures; their chemical compositions are very similar and so is the molecular biology of their interactions with permissive and nonpermissive host cells.

To date, SV40 has not been associated with any disease in adult monkeys, and its relationship with its natural hosts closely parallels the relationship between polyoma virus and the mouse. It would, of course, be rash to say categorically that SV40 never causes cancers in monkeys, but it seems most unlikely that the virus is an important carcinogen of its natural host.

Permissive versus Nonpermissive Cells

Cells that support the complete replication of polyoma virus or SV40, and as a result suffer lysis, are called permissive cells, whereas cells that do not support the replication of these viruses but may be stably transformed by them are called nonpermissive cells. Some cells (e.g., mouse cells, including those of stable lines such as 3T3) are nonpermissive for SV40 but permissive for polyoma virus; other cells (e.g., hamster cells) do not allow either virus to replicate well. However, cells permissive for both SV40 and polyoma virus have never been described.

We do not know why some cells are permissive and others are nonpermissive. Hybrid cells formed by fusing permissive mouse cells and nonpermissive hamster cells are productively infected by polyoma virus; the permissiveness of such hybrids to infection by polyoma virus is a dominant character contributed by the murine chromosomes (Basilico 1971). This result suggests that permissiveness may be the consequence of the production by mouse cells of a factor or factors necessary for the replication and/or release of polyoma virus. Hamster cells are presumably nonpermissive, not because they synthesize repressors that

specifically inhibit the multiplication of polyoma virus and SV40, but because they fail to make the necessary permissive factor(s). The nature of these putative factors and how they act are unknown.

Because permissive cells are killed by SV40 and polyoma virus, they can be used in plaque-assay tests to titrate the number of infectious particles in stocks of these viruses. Confluent monolayers of cells are first infected with various dilutions of virus, and then they are overlayed with medium containing about 0.9% agar. After incubation at 37°C for several days, the monolayers are stained with neutral red, a dye that is taken up only by living cells. Viral plaques appear as unstained areas in a background of stained cells. The total number of viral particles in a preparation of virus can be counted by electron microscopy; it exceeds the number of infectious units by a factor of 100 or more.

With both polyoma virus and SV40 it is possible to isolate the very rare transformed cell from populations of productively infected permissive cells. For example, mouse 3T3 cells transformed by polyoma virus (Py3T3) and African green monkey kidney cells transformed by SV40 (SV-AGMK) have been isolated. These rare transformants are thought to arise either from permissive cells infected by a defective viral particle that cannot complete its replication but is capable of transforming, or from rare variant cells in the population that cannot support a productive infection.

Rescue of SV40 from Transformed Cells

For several years, much uncertainty surrounded the observation that cultures of cells transformed by SV40 often yielded a few infectious SV40 particles. Because polyoma virus never could be recovered from the cells it transforms, airborne contamination was considered a distinct possibility. Another explanation seriously proposed was that populations of nonpermissive cells may regularly give rise to a very small fraction of permissive variants. By postulating that each time a nonpermissive cell divides there is a very low but finite probability that one of the daughter cells will be a permissive variant able to support the

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Simian virus 40 infection via MHC class I molecules and caveolae

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Summary: MHC class I molecules are a necessary component of the cell surface receptor for simian virus 40 (SV40). After binding to class I molecules, SV40 enters cells via a unique endocytic pathway that involves caveolae, rather than clathrin-coated pits. This pathway is dependent on a transmembrane signal that SV40 transmits from the cell surface. Furthermore, it delivers SV40 to the endoplasmic reticulum, rather than to the endosomal/lysosomal compartment, which is the usual target for endocytic traffic. The glycosphingolipid and cholesterol-enriched plasma membrane domains that contain caveolae are also enriched for class I molecules, relative to whole plasma membrane. Nevertheless, although class I molecules bind SV40, they do not enter with SV40, nor do they enter spontaneously into uninfected SV40 host cells. Instead, they are shed from the cell surface by the activity of a metalloprotease. These results imply the existence of a putative secondary receptor for SV40 that might mediate SV40 entry. It is not yet clear whether class I molecules are active in transmitting the SV40 signal. Monoclonal antibodies against class I molecules also induce a signal in the SV40 host cells. However, the antibody-induced signal is mediated by mitogen-activated protein kinase (MAP kinase), whereas the SV40 signal is independent of MAP kinase.

Introduction

Viruses initiate infection by binding to their specific receptor on the surface of a susceptible host cell. MHC class I molecules are the specific cell surface receptor for simian virus 40 (SV40) and are necessary for SV40 binding that leads to infection (1, 2). Viruses enter cells by either of two general mechanisms (3). Some enveloped viruses (e.g. HIV) enter by direct fusion of their envelopes with the plasma membrane. Other enveloped viruses (e.g. influenza), as well as non-enveloped viruses (e.g. SV40, adenoviruses), enter cells by endocytosis. The most frequently described and best understood endocytic pathway is that which occurs constitutively through clathrin-coated vesicles (3, 4). Nevertheless, some viruses appear to enter in non-coated vesicles via pathways that are not well understood. These viruses include polyoma virus (5), canine parvovirus (6), and SV40 (7, 8). The non-coated vesicles that mediate SV40 entry were recently shown to have the biochemical and morphological features of caveolae (9, 10), and SV40 is thus far the only

virus known to enter through caveolae. SV40 entry is also unusual with respect to its dependency on a transmembrane signal that SV40 transmits from the cell surface (11, 12). Also, SV40 is targeted to the endoplasmic reticulum (ER) (8, 13), rather than to the endosomal/lysosomal compartment, which is the usual target for endocytic traffic. The coordination of signal transmission with virus entry, and the role of class I molecules in SV40 signaling and entry, are discussed below.

Background

SV40 is a member of the Polyomaviridae family (14), and its natural host is the Asian rhesus macaque. The family also includes lymphotropic papovavirus of the African green monkey, JC and BK viruses of humans, and the mouse polyoma virus. The latter virus and SV40 are the best understood of the Polyomaviridae.

SV40 was first discovered in the early 1960s as a frequent contaminant of the rhesus kidney cell cultures that were then used to grow virus vaccines (15). By the time of its discovery, SV40 had been inadvertently administered to hundreds of millions of humans via both the live and attenuated poliovirus vaccines. SV40 is generally not considered a human pathogen. However, it was recently shown using PCR technology that SV40 DNA is present in tumors of people some of whom were never exposed to SV40-contaminated vaccines (16). Thus, it remains possible that SV40 is pathogenic in humans. Furthermore, SV40 may circulate in the human population.

The SV40 genome, like that of the other Polyomaviridae, contains about 5,240 base pairs in a covalently closed superhelical DNA molecule (14). It is divided into an early region and a late region. The early region encodes the large and small T antigens and the late region encodes the capsid proteins VP1, VP2, and VP3. These regions are separated by a regulatory region that also contains the origin of DNA replication. The SV40 capsid is a non-enveloped 40 nm icosahedron that contains 360 molecules of VP1 and about 20 to 30 molecules each of VP2 and VP3. VP1 is organized into 72 pentameric capsomers, whereas the organization of VP2 and VP3 within the capsid is not clear.

The only cell surface receptor for a polyomavirus that has been identified to date is that for SV40. However, all known polyomaviruses other than SV40 interact with sialic acids on the cell surface. Consequently, those polyomaviruses, but not SV40, are able to hemagglutinate red blood cells.

SV40 and polyoma virus first attracted great interest when they were found to induce tumors in laboratory animals and neoplastic transformation of cells in culture. Another important but frequently overlooked feature of the Polyomaviridae is that they give rise to life-long persistent infections in their natural hosts (17).

Earlier, our laboratory studied a model system of rhesus kidney cells, and later of human glial cells, that were persistently infected with SV40 (e.g. (17, 18)). Our purpose was to elucidate the viral and cellular factors that enable this cytopathic virus to establish and maintain persistent infections of permissive cells in culture. Note that these systems of persistent infection are fundamentally different from systems of neoplastic transformation. The former systems involve cells that support lytic virus replication, whereas the latter involve cells that do not.

In brief, we found that whereas virtually all cells of stable persistently infected cultures express SV40 T antigen, only a small subset of cells produce virus at any time (17). The virus-producing cells are killed, and new virus-producing cells continually emerge from the non-producing population to maintain the persistent infection. Since virtually all cells in the persistently infected cultures express SV40 large T antigen, and since SV40-specific cytotoxic T lymphocytes (CTLs) respond to T-antigen expression in an MHC I-restricted manner (19), we were interested in how similar cells *in vivo* might evade host antiviral defenses. This led us to ask whether the level of MHC I molecules might actually be diminished at the surfaces of SV40-infected cells. As described below, our initial inquiries in this regard led to the identification of class I molecules as the cell surface receptor for SV40 and our current studies. Note that it was shown by others that SV40 actually upregulates class I expression (20, 21). Furthermore, the expression at the cell surface of either higher or lower levels of class I molecules is not a major determinant of the tumor-inducing capacity of SV40-transformed cells *in vivo* (21).

MHC I molecules are a necessary component of the SV40 receptor

As noted above, the initial purpose of our inquiry into the interaction of SV40 with the MHC was to determine whether SV40 might affect the expression of class I molecules at the cell surface. Using a radioimmunoassay to measure the levels of surface class I molecules, we indeed detected diminished binding of anti-HLA monoclonal antibodies (MAbs) to SV40-infected rhesus kidney cells at 7 days. However, when the cultures were washed and passaged, the original preinfection levels of surface class I molecules appeared to be restored. We knew from earlier thin-section electron microscopy that progeny SV40 particles accumulate on the surfaces of infected cells

(22). Together, these results suggested that the accumulation of progeny SV40 particles on the cell surfaces might have been blocking the binding of the anti-HLA antibodies in our radioimmunoassays. Considering this possibility, we found that preadsorbing SV40 to cells (in the cold to prevent entry) indeed blocks the binding of anti-HLA. Furthermore, the antibody-blocking affect of SV40 was specific for anti-HLA, since preadsorbed virus had no affect on the binding of control MAbs against either lymphocyte function-associated molecule 3 or the fibronectin or vitronectin receptors. This suggested that the interaction between the progeny SV40 particles and surface class I molecules is specific.

We next asked whether class I molecules might actually be the cell surface receptor for SV40. We began by asking whether preadsorbing saturating amounts of each of several different anti-HLA MAbs to the host cells might block infection by SV40. Each of the anti-HLA MAbs, but none of the control MAbs, were found to block infection. The abilities of the anti-HLA MAbs to block infection was greatly reduced when the order of addition of the MAbs and the virus was reversed (1).

In the experiments noted above, we measured the abilities of the antibodies to block infection (as measured by immunofluorescent staining for SV40 T antigen at 48 h), rather than merely to block binding. This was done since we wanted to know whether binding to MHC I actually leads to infection. In other experiments, we asked whether the various antibodies block SV40 binding, as measured by flow cytometry. As expected, the anti-HLA MAbs, but not the control MAbs, block SV40 binding (2). Also, SV40 binds specifically to a limited number of receptors, as shown by the ability of an excess of unlabeled virus to compete with labeled virus for binding (2). An earlier Scatchard analysis implied that SV40 binds to a single class of receptors (23). Taken together, these findings show that MHC class I molecules are the functional SV40 receptor, mediating binding that leads to infection.

In other experiments we found that both binding and infection are impaired by preincubating SV40 with purified soluble HLA (1, 2). Also, if the purified HLA is preincubated with the virus, it can then be immunoprecipitated by anti-SV40 antiserum (2). These results show that SV40 can attach directly to MHC I molecules, rather than to another class of molecules that might be associated with MHC I. To confirm the specificity of this interaction, we found that incubating SV40 with proteins extracted from the cell surface, followed by immunoprecipitation with anti-SV40 antiserum, resulted in the selective immunoprecipitation of MHC I molecules (2).

Two cell lines that do not express surface MHC I molecules were used to show that class I molecules are indeed an essential component of the SV40 receptor (2). Daudi cells do not express surface MHC I molecules because of a defect in the gene for β_2 microglobulin. The other line, a derivative of the .221 line of human B-lymphoblastoid cells, does not express the three major human classical transplantation antigens, HLA-A, HLA-B, and HLA-C, because of γ-ray-induced mutations in the HLA complex. SV40 does not bind to cells of either line. However, SV40 binds to Daudi cells after they are transfected with the cloned gene for β_2 microglobulin. Similarly, SV40 binds to the .221 derivative only after it has been transfected with a cloned HLA gene. Surface MHC-I expression is also restored in the transfectants. SV40 binding to the HLA-A, HLA-B, HLA-C mutant cells and to the HLA transfectant line is shown in Fig. 1. Note that the sialic acid-dependent polyomaviruses, including polyoma virus, JC virus, and lymphotropic papovavirus, do not use MHC I molecules for their receptors (23–26). Thus, the Polyomaviridae do not necessarily share the same receptor, as is the case for other virus families (27).

The SV40 entry pathway

The SV40 entry pathway differs in several important ways from the known entry pathways of other viruses that also enter by endocytosis. The unusual aspects of SV40 entry are described below.

SV40 entry is notably slow

We measured the rate of bulk virus clearance from the cell surface and, also, the time course for virus entry that leads to infection. Bulk virus clearance was measured by flow cytometry. Our assay for the rate of SV40 entry leading to infection was based on our finding that SV40 remains sensitive to neutralization by anti-SV40 antiserum while the virus is at the cell surface (11). Thus, the time required for preadsorbed virus to enter cells can be determined, since infection would then become resistant to neutralization by added antiserum. Individual infected cells are detected by immunofluorescent staining for T antigen at 48 h. Our assays for bulk entry and for infectious entry each showed that SV40 does not enter until about 1.5 h after it is preadsorbed to cells (9). In control experiments, we found that transferrin (a ligand known to enter via receptor-mediated endocytosis) is internalized within 4 min of preadsorption.

SV40 is targeted to the endoplasmic reticulum

The majority of entering SV40 particles are targeted to the ER, as shown by an earlier electron microscopy study (8). More recently, using double-label confocal microscopy, we obtained results that agreed with the earlier report (13).

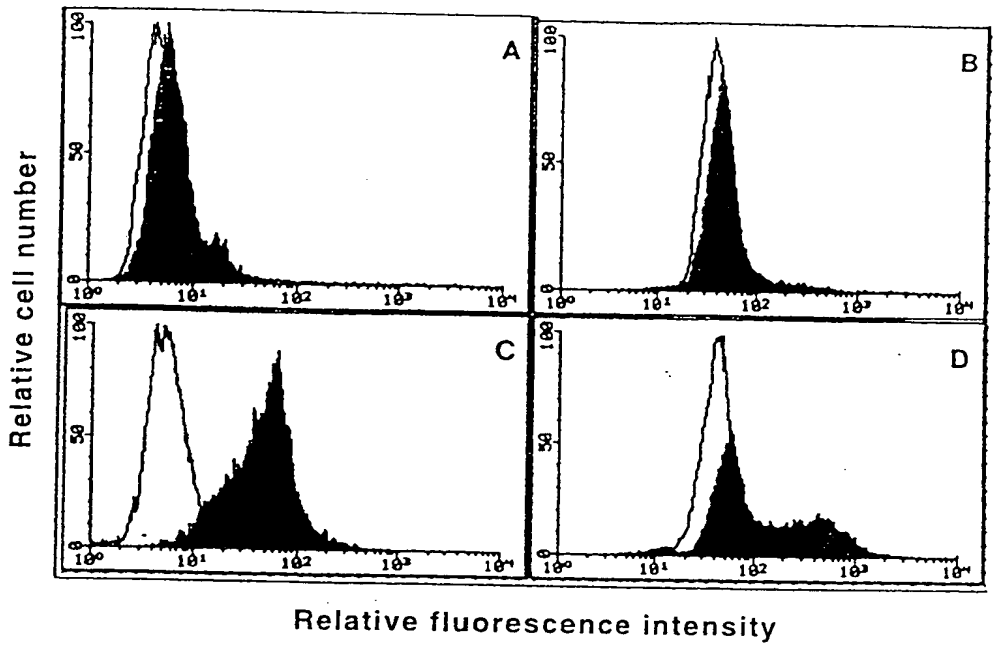

Fig. 1. Expression of class I HLA and the SV40 receptor on the *HLA-A, HLA-B, HLA-C null* mutant 976.1 cells and on the HLA-B8 transfectant line pHPT(B8)-.221. Expression of surface HLA and SV40 binding were assessed by flow cytometry. HLA was detected with anti-HLA mAb BB7.7 by using a fluorescein isothiocyanate (FITC)-labeled secondary antibody. SV40 was detected by using rabbit anti-SV40 sera and FITC-labeled goat anti-rabbit IgG. **A, B. BB7.7 binding (A) and SV40 binding (B) to 976.1 cells. C, D. BB7.7 binding (C) and SV40 binding (D) to pHPT(B8)-.221 cells.** Open peaks, control fluorescence of cells reacted with FITC-labeled secondary antibody only (A, C) and with rabbit anti-SV40 sera plus FITC-labeled goat anti-rabbit IgG (B, D); shaded peaks, binding of BB7.7 and SV40 as indicated above.
[Reprinted with permission of Breau WC, Atwood WJ, Norkin LC. Class I major histocompatibility proteins are an essential component of the simian virus 40 receptor. J Virol 1992;66:2037–2045. Copyright 1992. The American Society for Microbiology.]

The targeting of SV40 to the ER is unusual since the endosomal/lysosomal compartment is generally the target for endocytic traffic, including viruses that enter by endocytosis. The endosomal/lysosomal tropism of viruses is functionally significant, since the low pH of that compartment generally plays a major role in the uncoating and entry into the cytoplasm of lysosomatropic viruses (4). In contrast, infection by SV40 is not dependent on acidic compartments, as shown by the insensitivity of SV40 infection to lysosomatropic drugs (28).

SV40 entry is promoted by a transmembrane signal

SV40 induces a transmembrane signal from the cell surface, as indicated by the upregulation of cellular early response genes (e.g. *c-myc* and *c-jun*) within 30 min of prebinding SV40 in the cold (11). The response is independent of viral gene expression and cellular protein synthesis. Also, the signal is mediated by a tyrosine kinase and by protein kinase C, as implied by its sensitivity to specific inhibitors.

We do not know whether the upregulation of early response genes *per se* is important for SV40 replication. This is possible since the upregulation of early response genes is associated with the passage of quiescent cells into the G_1 phase of the cell cycle and SV40 replication is dependent on cellular enzymes for DNA replication that are only present in cycling cells. Although the SV40 T antigens also stimulate resting cells to enter the cell cycle (14), the early SV40 transmembrane signal might prime cells to support virus replication.

Regardless of whether the SV40-induced signal affects SV40 replication, it does promote SV40 entry into the cell. This was initially shown by our finding that infection remains sensitive to

Fig. 2. Cross-sectioned cultured mouse embryo cell 24 h after infection with SV40. There are no adsorbed virions; all are membrane enveloped and partially internalized. The rough endoplasmic reticulum is intact with ribosomes. Magnification: ×100,000.
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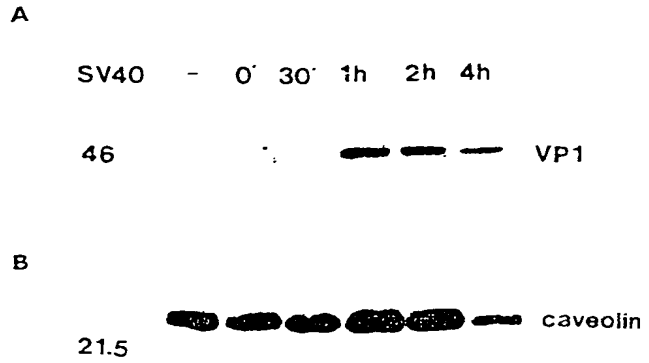

Fig. 3. Time course of SV40 translocation to the caveolin-enriched low-density Triton-insoluble membrane fraction. CV-1 cells were infected with SV40 (100 plaque-forming units/cell) for 1 h on ice and harvested at the indicated times after incubation at 37°C. The low-density detergent-insoluble complexes were isolated and analyzed by Western blotting. The same membrane was cut and immunoblotted against SV40 capsid protein VP_1 (A) and against caveolin (B).
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neutralization by anti-SV40 antiserum while signaling is blocked by the tyrosine kinase inhibitor genistein (11). Neither genistein nor anti-SV40 antiserum affect SV40 infection when added after SV40 entry. The effect of genistein on infection is reversible, provided that neutralizing antiserum is not added prior to SV40 entry. The effect of the drug is specific for the SV40 entry pathway since it has no effect on the internalization of transferrin. Thus, when signaling is blocked, SV40 remains exposed at the cell surface and is susceptible to neutralization by antiserum.

SV40 enters through caveolae

Caveolae are distinctive flask-shaped invaginations of the plasma membrane that are about 70 to 100 nm in diameter (see (29, 30) for recent reviews). They are distinguished from clathrin-coated pits by their characteristic size and shape and by the presence of caveolin, an integral membrane protein that serves as a caveola marker (31). Caveolae are also highly enriched for glycosphingolipids and cholesterol. Consequently, they partition into a low-density Triton X-100-insoluble cell fraction. The precise functions of caveolae are not yet entirely clear, but they have been implicated in endocytosis, transcytosis, intracellular signaling, and an endocytic process called potocytosis.

The possibility that SV40 entry might occur via caveolae was suggested to us by the resemblance between the small non-coated surface invaginations that were earlier seen to mediate SV40 entry (7) and structures that might now be recognized as caveolae (Fig. 2). In our first experiments to ascertain whether SV40 might enter through caveolae, we assayed for infectious SV40 entry under conditions that are known to selectively impair caveolar function (9). Nystatin is a cholesterol-binding drug that removes cholesterol from membranes. Since caveolae are highly enriched for cholesterol, nystatin can selectively disrupt caveolae while not affecting clathrin-coated pits or other submembraneous structures (31, 32). Prolonged treatment of cells with the phorbol ester phorbol myristate acetate (PMA) also selectively affects caveolae (33). When these drugs are present immediately after preadsorption of SV40, infection remains sensitive to neutralization by anti-SV40 antiserum. The effect of the drugs on infection is reversible, provided that antiserum is not added prior to virus entry. Thus, nystatin and PMA cause preadsorbed SV40 particles to remain at the cell surface, where they are susceptible to neutralization by antiserum. In control experiments, SV40 entry was not blocked by cytosol acidification, a standard treatment for blocking endocytosis through clathrin-coated pits. Also, transferrin internalization was insensitive to both PMA and nystatin, but was sensitive to cytosol acidification. Thus, PMA and nystatin were specific for the SV40 entry pathway.

As noted above, caveolin is highly enriched in low-density Triton-insoluble complexes (LDTICs) that can be purified by flotation in sucrose density gradients. We found that whereas little if any preadsorbed SV40 is initially associated with the LDTICs, maximal levels of SV40 become associated with that

membrane fraction between 30 min and 1 h (Fig. 3) (9, 12). Nystatin does not block SV40 binding, but does prevent the association of the virus with the LDTICs. The above results imply that SV40 initially binds to flat regions anywhere on the plasma membrane and later associates with caveolae-containing membrane domains. This was confirmed by Stang and co-workers using immunoelectron microscopy (10) and by our analysis of negatively stained LDTICs (12).

The SV40 signal promotes SV40 enclosure within caveolae

In more recent experiments we considered how the SV40 signal might be coordinated with the caveolae-mediated SV40 entry pathway (12). First, genistein does not prevent the translocation of SV40 from its initial binding sites to the caveolin-enriched membrane domains. Thus, the signal is required for a later step in the entry pathway. Second, SV40 signaling is specifically impaired by nystatin, implying that the signal is actually transmitted from the cholesterol-enriched caveolae-containing membrane domains. Less likely, perhaps, is that a signaling factor is recruited from those domains.

Electron microscopy of negatively stained LDTICs showed that SV40 particles accumulate at the annuli or mouths of caveolae when genistein is present (12). This finding implies that the SV40-induced signal is required for the enclosure of SV40 within caveolae. Representative samples of negatively stained LDTICs are shown in Fig 4. Electron microscopy of thin sections was in agreement with that of the negatively stained LDTICs, showing a much lower percentage of surface SV40 particles enclosed within caveolae when genistein is present.

Stang and co-workers (10) found by immunoelectronmicroscopy that the caveolin density around the SV40-containing caveolae increases with time. This result is consistent with their suggestion that SV40 particles might act as nucleation centers for the formation of new invaginated caveolae. However, our analysis of the LDTICs showed SV40 at the mouths of caveolae in both genistein-treated and control cultures, and the virus was found to accumulate at the mouths when genistein was present (Fig. 4) (12). Thus, the caveolar mouths appear to be the entry site for SV40 into pre-existing caveolae, and the signal may facilitate the entry process. The signal might promote SV40 entry into caveolae by causing the rigid caveolar necks to relax so that the virus might pass, or perhaps by creating a targeting signal for caveolae on the virus's secondary receptor (see below).

MHC class I molecules are enriched in caveolae but do not enter with SV40

In general, little is known about the fate of virus receptors after virus has been bound. The fate of MHC I molecules after SV40 binding was particularly interesting because of the attractive possibility that the atypical targeting of SV40 to the ER might result from a unique endocytic pathway that recycles surface MHC I molecules back to the ER (i.e. the site where MHC I molecules acquire antigenic peptides for presentation at the cell surface (34)). A number of earlier studies, mostly involving T cells, have asked whether surface MHC I molecules might internalize. However, there does not appear to be a consensus regarding this issue (see (34, 35) for further discussion and references).

Before asking whether class I molecules might internalize with SV40, we asked whether they might mediate the translocation of SV40 from its initial binding sites in the detergent-soluble membrane regions to the detergent-insoluble caveolin-enriched membrane domains. At first, we could not detect any class I molecules in the caveolin-enriched membrane fraction. This was because the class I molecules are extracted by the Triton X-100, as shown by the abundance of class I molecules in the caveolin-enriched fraction when it is prepared using a detergent-free procedure (35). Indeed, when the caveolin-enriched fraction is prepared by the detergent-free method it is also enriched for class I molecules, relative to highly purified whole plasma membrane prepared using a silica coating-based procedure (35).

The enrichment of the caveolae-containing membrane domains for class I molecules is consistent with the likelihood that SV40 translocates from flat regions of the plasma membrane to caveolae in association with class I molecules. However, exposing cells to saturating amounts of SV40 did not lead to an observable increase in the amount of class I molecules in the caveolin-enriched membrane fraction (35). Nevertheless, it is possible that any SV40-induced increase in the already high basal levels of class I molecules in the caveolin-enriched membrane domains might have been below the sensitivity of our procedures.

We next asked whether MHC I molecules might internalize with SV40. This was done by first surface-labeling the host CV-1 cells with ^{125}I. The cells were then exposed to SV40 for various periods of time before treating them with papain, which cleaves class I molecules at a specific site just proximal to the plasma membrane. No papain-resistant (i.e. internalized) surface-labeled class I molecules were detected at any time in either control cells or in cells that were exposed to saturating amounts of SV40. Instead, the surface-labeled class I molecules accumulate in the medium, coincident with their turnover from the cell surface. The class I heavy chains that accumulate in the medium are truncated to about 37 kDa and their release is specifically prevented by the metalloprotease inhibitor 1,10-phenanthroline (see Concluding Remarks) (35). Other results implied that the heavy chains are cleaved only after the β2 microglobulin moieties are spontaneously released.

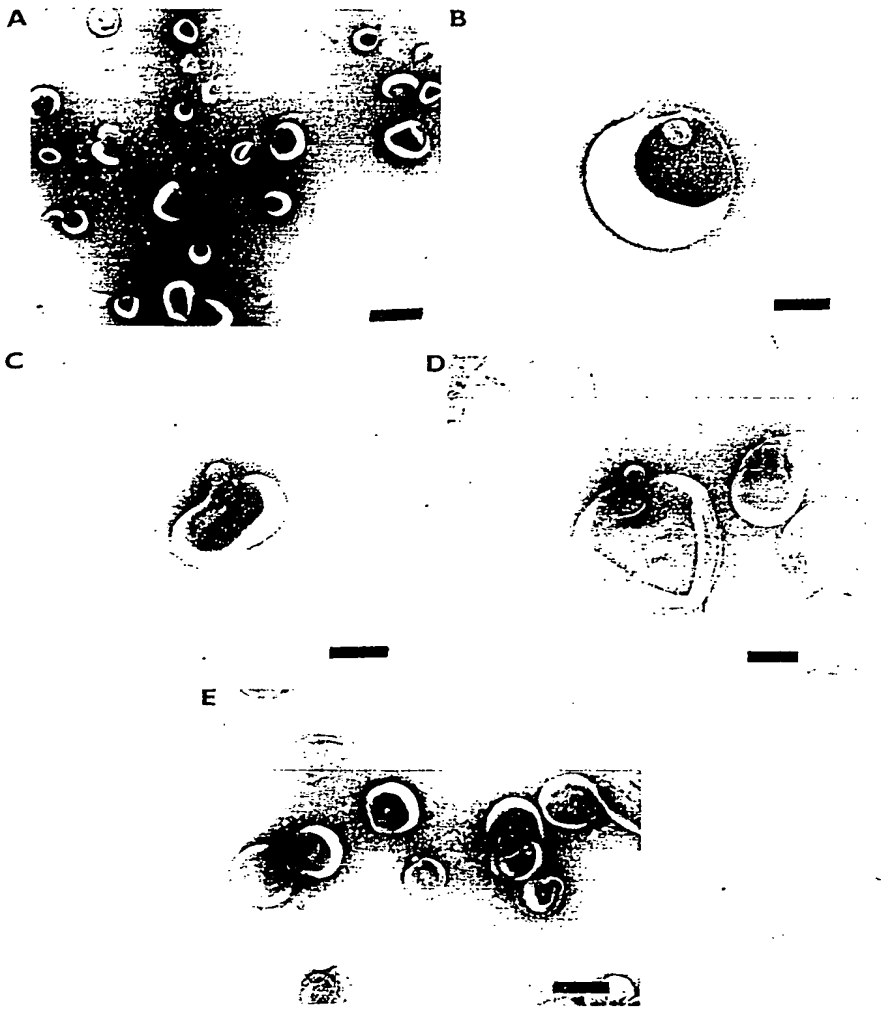

Fig. 4. **Negative staining of the low-density Triton X-100-insoluble complexes isolated from CV-1 cells.** Caveolae-like vesicles were typically attached to the inside of larger spherical vesicles. Similar structures have been seen in caveolae fractions isolated from other cell types (46, 53–55). The outer membranes of these complexes may arise from the flat regions of the Triton X-100-insoluble membrane domains during extraction.
A. Complexes from untreated uninfected cells.
B. Complex from SV40-infected cells treated with ethanol (the genistein carrier); note the virion enclosed within the inner vesicle.
C, D. Complexes from SV40-infected cells treated with genistein (200 μM); note the virions at the mouths of the caveolae.
E. Complexes from SV40-infected cells treated with ethanol; note the virions at the mouths of the caveolae. Cells were exposed to SV40 (100 plaque-forming units/cell) for 1 h on ice and then incubated for 1 h at 37°C. Virions are recognized by their shape and electron density. Bars: 500 nm in A; 100 nm in B, C, and D; 200 nm in E.
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We also followed the fate of surface MHC I molecules that were indirectly labeled with ^{125}I-labeled anti-HLA MAbs. Consistent with our findings from the direct labeling approach, no papain-resistant ^{125}I-labeled anti-HLA could be detected at any time. Instead, the labeled antibody was also seen to accumulate in the medium, coincident with its turnover from the cell surface. Identical results were obtained regardless of whether the bound anti-HLA was cross-linked with a secondary antibody. In control experiments, virtually all preadsorbed ^{125}I-labeled transferrin became papain-resistant within 5 min of transferring the cells to 37°C. These results show that surface class I molecules on CV-1 cells do not spontaneously internalize. Furthermore, whereas class I molecules mediate SV40 binding, they do not mediate SV40 entry.

Concluding remarks

The unusual features of the SV40 entry pathway include its slowness, its dependency on a transmembrane signal, and the targeting of SV40 to the ER. Each of these features might be

related to the central role of caveolae in the SV40 entry pathway. For example, the slow rate of entry probably results, at least in part, from the slow migration of the virus–receptor complexes to the caveolar domains, and from the dependency of entry on a signal transmitted from those domains.

Since the targeting of a ligand from the plasma membrane to the ER is unusual, it is reasonable to ask what benefit SV40 might derive from its atypical entry pathway. One possibility is that this pathway might facilitate the entry of SV40 into the cell nucleus, the site of its replication (14). Intact input polyomaviruses have been observed in the nucleus (5), even though the Polyomaviridae are about twice the diameter of the largest particles that can pass through nuclear pores by facilitated transport. The SV40 entry pathway via the ER might facilitate SV40 entry into the nucleus since the ER membranes are continuous with the outer nuclear membrane and the space between the nuclear membranes is continuous with the ER lumen. Nevertheless, entry of SV40 into the ER lumen still leaves the question of how the virus might cross the inner nuclear membrane. Also, SV40 particles appear able to enter the nucleus from the cytoplasm, as suggested by experiments in which intact virions were microinjected into the cytoplasm (36).

An alternative benefit that SV40 might derive from its caveolae-mediated entry pathway is the facilitation of its dissemination in the intact host. This might result from the likely role of caveolae as the mediators of transcytosis across polarized endothelial cells (e.g. (37, 38)). Thus, the SV40 entry pathway might also enable the virus to pass from the circulation, across the vascular endothelium, to underlying tissue. In addition, uptake via a non-lysosomal pathway, that is transcytotic in endothelial cells, may enable the virus to avoid degradation in other cell types.

If the caveolae-mediated SV40 entry pathway indeed provides SV40 with a particular advantage, then class I molecules might be an advantageous receptor for SV40 because of their possible link to caveolae. The enrichment of the caveolae-containing membrane domains for class I molecules is consistent with that possibility. Regardless, by using class I molecules for its cell surface receptor, SV40 exploits a major factor of cellular immunity to gain access to its target host cells.

Our finding that surface class I molecules do not internalize into SV40 host cells suggests that SV40 entry is mediated by an as yet unknown secondary receptor. A variety of other viruses are indeed known to use different cell surface molecules to mediate the series of steps between binding and entry. Examples include non-enveloped viruses such as adenoviruses (39), and enveloped viruses such as herpesviruses (40) and HIV (41, 42) (reviewed in (43)).

The role of class I molecules in transmitting the SV40-induced signal is not yet clear. In support of a post-binding role for class I molecules in signal transmission, anti-HLA MAbs also induce the upregulation of early response genes in CV-1 cells (11). However, whereas the antibody-induced signal is transmitted by a mitogen-activated protein kinase (MAP kinase)-dependent signaling pathway, the SV40-induced response is independent of MAP kinase (11).

Signal transmission by class I molecules would seem to require the involvement of a co-factor, since class I molecules are not intrinsically linked to transmembrane signaling complexes. The differences in the signaling pathways induced by anti-HLA antibodies and SV40 might then reflect the recruitment of different signaling co-factors by each. However, since SV40 entry is likely mediated by a secondary receptor, it is possible that SV40 signal transmission is also mediated by the secondary receptor.

We do not know whether the antibody-induced signal in CV-1 cells represents a physiologically relevant process. One possibility is that it might reflect a signal transmitted by a CTL into a target cell, resulting from the interaction of the T-cell receptor complex on the CTL with an MHC-I/peptide complex on the target cell. With that possibility in mind, we asked whether the anti-HLA-induced signal might induce apoptosis or the upregulation of FAS, a member of the tumor necrosis factor/nerve growth factor receptor family which transduces an apoptotic signal upon binding the Fas ligand (43). It did neither (Y. Chen, L. C. Norkin, unpublished results).

The following model for SV40 entry is consistent with our findings. First, SV40 binds to MHC I molecules that are mainly on flat regions of the plasma membrane. Then, in association with class I molecules, SV40 translocates to the glycosphingolipid and cholesterol-enriched membrane domains that contain the caveolae invaginations. In the flat portions of those membrane domains SV40 dissociates from MHC I molecules and attaches to its putative secondary receptor. The SV40 signal is then transmitted, promoting SV40 entry into caveolae. The signal might also promote later stages of SV40 entry, such as caveolar budding. Note that the flat regions about the caveolae, as well as the caveolar invagination *per se*, are enriched for a variety of signal-transducing molecules (e.g. (44–46)).

Our findings concerning the fate of surface class I molecules (35) are consistent with reports that surface class I heavy chains are cleaved by a metalloprotease after the spontaneous release of the peptide and the β_2 microglobulin moiety (47–49). The instability of empty MHC-I heterodimers and the cleavage of free heavy chains from the cell surface is believed to prevent the rebinding of peptide by bystander cells (e.g. (48, 49–52)).

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Exploitation of major histocompatibility complex class I molecules and caveolae by simian virus 40

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Summary: Simian virus 40 (SV40), a non-enveloped DNA virus, is transported from the cell surface to the nucleus where virus replication occurs. This pathway of virus uptake involves binding to surface MHC class I molecules, entry via non-coated pits, and subsequent transport to the endoplasmic reticulum (ER). At some stage in this pathway the virus must cross a membrane to reach the cytosol. In the present review, the cellular machinery which the virus has utilized to enter the cell will be examined. In particular, we will consider recent evidence for the involvement of caveolae in the infectious entry step and propose a model involving recruitment of caveolar proteins around the membrane-bound virus. We also speculate that a similar mechanism may have been exploited by bacterial pathogens. The subsequent steps by which SV40 reaches the ER remain unclear but recent evidence suggests that this pathway may be shared with several other proteins that are transported from surface caveolae to the ER.

Introduction

Animal viruses have evolved complex schemes to circumvent the protective barrier provided by the mammalian cell plasma membrane and to gain entry into their host cells. Despite the importance and wide variety of viral infections afflicting mankind today, the exact means by which many viruses penetrate host cell defences are unknown. A good example of this is simian virus 40 (SV40), a papovavirus, which has been extensively studied as a model system for cell transformation. The infectious properties of SV40 and the simplicity of the virus have also attracted the interest of investigators attempting to introduce foreign genes into human cells. Indeed, in recent years, SV40-based vectors have become of increasing importance as tools for gene therapy (1). It is therefore of fundamental importance to determine how SV40 enters animal cells and reaches its site of replication. SV40 is a simple non-enveloped DNA virus containing only three different virally encoded proteins, VP1, VP2 and VP3. This primitive virus has apparently exploited a component of the immune system, major histocompatibility complex (MHC) class I, to gain entry into the cell and to reach its site of replication. This review will consider recent studies which provide new insights into the process by which SV40 binds to, and

enters, mammalian cells. We will concentrate on the initial entry step but also consider recent studies relevant to the intracellular trafficking of the virus.

The SV40 infectious entry pathway

The first step in SV40 infection involves association of the viral protein VP1 with a cell surface protein, postulated to be MHC class I. This interaction has been demonstrated by a series of experiments, including inhibition of binding and infection by specific monoclonal antibodies and reconstitution of interactions between VP1 and MHC class I molecules *in vitro* (2–4). Moreover, cells lacking class I expression do not bind SV40, but binding activity can be restored by introduction of proteins which rescue the MHC class I defects (2). At the very least, this suggests that MHC class I is part of the receptor involved in binding SV40 at the surface of these cells. However, there is also evidence for a different surface receptor. In polarized epithelial cells, SV40 binding and internalization only occurs at the apical surface, a distribution which does not correlate with that of MHC class I (5).

The subsequent step in the virus infection pathway leading to the nucleus involves the endocytosis of SV40 particles into a membrane-bound compartment. The best-characterized endocytic pathway for virus entry, which has been particularly well-characterized for enveloped viruses such as Semliki Forest virus, involves receptor-mediated endocytosis via clathrin-coated pits (6). The pits bud off to form coated vesicles, which fuse with the early endosome. The low lumenal pH induces the virus to fuse with the endosomal membrane, allowing the nucleocapsid to reach the cytosol (7). This scheme differs significantly from the present view of SV40 entry. Morphological studies suggest that after binding to the cell surface, virus particles are internalized via uncoated invaginations (8). The virus-containing invaginations bud into the cytoplasm to produce vesicles that apparently fuse directly with the endoplasmic reticulum (ER) (8). The crucial step in the pathway to the nucleus involves the passage of the virus from the lumen of some membranous compartment (possibly the ER) into the cytoplasm. It is this step which is the least understood and yet perhaps the most interesting in the entry pathway, as the virus must cross a lipid bilayer. Although the mechanism is poorly understood, all transfection and gene therapy applications presumably rely on a similar type of transmembrane transfer step. A number of pieces of evidence suggest that this transport step results in intact virus reaching the cytoplasm. For example, virus can be microinjected directly into the cytoplasm and still cause infection (9–11). The virus is subsequently transported through the nuclear pore complexes from the cytoplasm to the nucleus. If nuclear import of the virus is blocked experimentally, virus particles do not reach the nucleus but accumulate in, or close to, the nuclear pores (11). Cytoplasmically microinjected antibodies to VP1 and VP3 also block SV40 infection, showing that these antigens are exposed in cytoplasm in the infectious entry pathway (10). Upon reaching the nucleus the viral DNA is released from the virion coat. Virus replication depends on expression of T-antigens, and it is the non-specific triggering of DNA replication (both host and virus DNA) which causes uncontrolled cell division.

SV40 entry and endocytosis

The suggested entry pathway for SV40, from the cell surface directly to ER, shares little resemblance to the classical membrane trafficking pathways in mammalian cells. This has raised the question of whether SV40 induces its own specialized internalization pathway. In this model, the plasma membrane would zip up around the virus through interactions between the viral VP1 proteins and the host cell receptors and then close to allow the virus to bud into the cell. Alternatively, SV40, like other viruses, may simply hijack some existing cellular endocytic machinery, just as Semliki Forest virus has exploited clathrin-coated pits (6).

Early morphological studies tended to rule out a role for clathrin-coated pits in SV40 uptake (8, 12, 13). Rather than the characteristic 100–120 nm clathrin-coated pits, the vehicles apparently mediating SV40 uptake were identified as uncoated pits of 45–55 nm diameter that form a tightly fitting membrane around the virus. This membrane is so closely apposed to the virus particle as to effectively exclude fluid phase markers from internalization with the virus in the same vesicles (8). One possibility for SV40 entry was the involvement of plasma membrane domains termed caveolae. Caveolae are 55–65 nm diameter uncoated pits with a unique protein and lipid composition (14, 15). Caveolae have been implicated in a number of endocytic processes (16, 17) and so could theoretically act as vehicles for viral entry. The internal diameter of a caveola is roughly comparable to the external diameter of an SV40 particle. In view of the evidence outlined above, the SV40 internalization pathway has been re-examined to investigate the possible involvement of caveolae. Before summarizing these studies, it is perhaps appropriate to briefly summarize some of the key features of caveolae.

Caveolae: 'small caves' with multiple functions

Over 40 years ago, electron microscopists described numerous 50–70 nm pits which covered the surface of mammalian cells

(18). These pits, now termed caveolae (small caves) are a characteristic and highly abundant feature of many mammalian cells, being particularly enriched in endothelial cells, smooth muscle, and adipocytes. Until recently, the study of caveolae was restricted to morphological descriptions. This changed with the discovery of a marker protein, termed VIP21 or caveolin (now called caveolin-1) (19, 20), which allowed detailed biochemical dissection. A wealth of molecules and functions have now been assigned to these enigmatic structures (15).

Caveolae represent surface domains with a distinct lipid composition (21) which depend on cholesterol for their integrity (22). These domains are also enriched in glycosphingolipids (23) and caveolins. Although caveolins are integral membrane proteins, both the N and C termini face the cytoplasm (14). Caveolin-1 is a palmitoylated protein which is present at high density in the caveolar membrane in the form of high molecular weight oligomers (24). Expression of caveolin-1 in a cell which normally lacks caveolae causes *de novo* formation of caveolae (25). Quantitative electron microscopic studies suggested that caveolin-1 plays a direct role in the formation of the caveolar invagination. Recently, two additional caveolin-family members have been described (14, 26, 27). While caveolins are generally regarded as markers of cell surface caveolae, caveolin-1 is also found in an intracellular pool associated with the Golgi complex (28). A pool of caveolin is known to cycle to the Golgi and return back to the surface in *trans*-Golgi network-derived exocytic vesicles (20). Increasing evidence implicates cycling of caveolin in cholesterol transport. Caveolin-1 binds cholesterol (29) and is redistributed to the ER upon cholesterol oxidation (30). These studies suggested a role for caveolin in cholesterol transport, a hypothesis supported by subsequent studies. Heterologous expression of caveolin-1 caused increased cholesterol transport between the ER and the cell surface (31). Further evidence for a tight link between cholesterol and caveolin was provided by the demonstration that caveolin-1 is regulated at the transcriptional level by cholesterol (32). Reduction of cellular cholesterol causes a reversible decrease in caveolin-1 and loss of surface caveolae (33). Subsequent studies showed that this regulation is mediated through cholesterol-responsive elements in the caveolin-1 promoter region (32). These studies clearly implicate caveolins and caveolae in the control of cellular cholesterol.

Caveolae have also been suggested to be sites of organised signal transduction events. A recent study showed that the entire MAP kinase activating pathway was associated with caveolae (34). Several studies have demonstrated direct functional interactions between a conserved 'scaffolding domain' of caveolin (residues 82–101 in caveolin-1) and a number of signalling molecules *in vitro*. These include alpha subunits of trimeric G proteins, the ras GTP binding protein, src kinases, nitric oxide synthase, and the epidermal growth factor receptor (35). In many cases, these interactions have only been shown *in vitro* and, in the case of trimeric G-protein interactions, their relevance has been directly questioned (36). Nevertheless, an increasing body of evidence suggests that caveolae are involved in at least some signalling events but in a negative capacity; caveolin generally has an inhibitory role to hold signalling molecules in an inactive conformation that is released upon receptor activation (35). Recent evidence suggests that cholesterol plays a crucial role in the organization of these signalling domains (37), perhaps linking the cholesterol transport and signal transduction properties of the caveolin molecule.

These properties of caveolae clearly distinguish them functionally from plasma membrane clathrin-coated pits, which are endocytic carriers with a defined life-time on the cell surface (approximately 1 min) before budding into the cytoplasm as coated vesicles. However, caveolae have also been implicated in endocytosis. In endothelia, caveolae bud from the cell surface to carry solutes across the endothelial monolayer (38–41). In fibroblasts and hepatocyte cell lines, caveolae may also play a role in endocytosis, particularly of glycolipids and glycosylphosphatidylinositol (GPI)-anchored proteins (16, 17, 23, 42). The former can be labelled with cholera toxin conjugates as the receptor for the toxin, the ganglioside GM1, is present in caveolae (23). Budding of caveolae may be regulated by phosphorylation at least in some cells (17). Like budding of coated pits, caveolae budding involves the large GTP-binding protein, dynamin, the mammalian homologue of the *Drosophila Shibire* gene (43, 44). Although caveolae were initially implicated in the uptake of small solutes such as folic acid in a specialized process termed potocytosis (45), a role in this process has been seriously questioned (46, 47).

SV40 and caveolae

In view of the postulated role of caveolae in endocytosis, the morphology of the virus-containing pits, and the intriguing observation that caveolin can cycle between the surface and the ER, caveolae were clearly an interesting candidate for mediating SV40 uptake. Employing a morphological approach using frozen sections and immunolabelling for SV40 and caveolin, it was shown that SV40 was concentrated within uncoated invaginations which were labelled with antibodies to caveolin-1 (4). Furthermore there was no detectable virus in clathrin-coated pits strengthening the case for the involvement of caveolae in this process. However, it was important to ascertain whether

caveolae actually represented the infectious entry pathway. Strong evidence for this possibility was provided by a series of independent experiments by Norkin and co-workers (48), who showed that agents which inhibit caveolae function, such as cholesterol-disrupting agents, blocked the infectious entry of the virus. The virus was also shown in these studies to associate with surface domains which are of low density and are detergent insoluble. These characteristics are typical, although not diagnostic, of caveolar domains (49) and have been termed detergent-insoluble glycosphingolipid-enriched domains (DIGs) or 'rafts' (21, 50). Moreover, our studies showed that specific antibodies against the putative SV40 receptor, MHC class I, both inhibited infection and prevented association of the virus with the caveolin-positive pits (4). Collectively, these studies link the infectious entry pathway to caveolae.

We further examined the possible role of MHC class I in this process. MHC class I molecules appear to be typical resident plasma membrane proteins with a dispersed distribution over the cell surface and a negligible endocytic rate (51). Intriguingly, earlier studies showed that surface labelling of MHC class I proteins via antibodies to β-1-microglobulin caused clustering in non-coated invaginations (52). We re-examined this in light of the above results and showed that antibodies to MHC class I caused clustering of the class I proteins and that these clusters were associated with caveolin-positive invaginations (4). This result suggests that MHC class I is a member of a group of surface proteins, including GPI-anchored proteins, beta adrenergic receptor, vesicular integral membrane protein (VIP) 36, and the influenza virus haemagglutinin protein, which upon cross-linking associate with caveolae or other plasma membrane DIG domains (28, 53, 54). It has been suggested that the individual proteins have relatively low affinity for these domains under physiological conditions but that the multimerization of the membrane components caused by cross-linking will co-operatively strengthen this association and so cause lipid domains to associate with the clusters (54). The cross-linking process is presumably mimicked by SV40 binding. It is likely that this property of MHC class I molecules has some physiological role, which has been exploited by SV40, but at present this role is unclear and the endocytosis of MHC class I molecules, even after cross-linking, has not been convincingly demonstrated (see below).

A model for SV40 entry: SV40-induced recruitment of caveolin

Taken together, the above data suggest a role for caveolae in SV40 entry. This could be a transient association in order to trigger signal transduction pathways known to be required for SV40 entry (55). However, the studies described above argue that caveolae are the actual vehicle mediating virus entry. This, however, may be an oversimplification. Morphological studies showed a clear size difference between 'empty' caveolae and those containing virus particles (Fig. 1) (4). Time-course studies also showed a gradual progression from shallow pits containing virus to deeply invaginated pits almost completely enclosing the virus; in contrast, empty caveolae are invariably flask shaped (i.e. the intermediates between shallow pits and vesicles are not obvious in caveolae as compared to clathrin-coated pits). In addition, parallel immunoelectron microscopy showed an increased density of caveolin labelling associated with the membrane surrounding the virus particle as the apparent invagination process progressed. Taken together these results argue against a simple model in which SV40 bound to clustered MHC class I molecules diffuses in the plane of the membrane and enters pre-existing caveolae. Rather, we favour a model in which the virus, presumably bound to MHC class I molecules, recruits caveolin around the virus particle to generate a caveola-like pit. This model is summarized in Fig. 2. This would explain the tight-fitting virus membrane around the virus and the size difference between occupied and unoccupied caveolae. This model could perhaps be considered similar to the budding of an enveloped protein out of a cell; in both cases interactions of virus components with the membrane leads to the deformation of the membrane and the recruitment of specific proteins. The budding of the virus-containing pit into the cell has been postulated to involve a specific phosphorylation event (55), perhaps analogous to that stimulated more non-specifically by the general phosphatase inhibitor, okadaic acid, which induces general caveolae internalization (17).

This model of SV40 uptake raises a number of questions; for example, what role does MHC class I play in this process? Does the virus-clustered MHC class I directly cause recruitment of caveolin? Does the virus stay associated with the MHC class I during the infectious entry process and get transported to the ER? While many of these questions remain unanswered, recent work suggests that MHC class I are not internalized with the virus but may act as a primary receptor to pass the virus to another, as yet undefined, surface component (56). It is also unclear which is the source of the caveolin recruited to the virus-associated membrane. This could be a pool of plasma membrane caveolin in equilibrium with the caveolar pool or could even be caveolin recruited from the cytosol, as recent studies have raised the possibility that caveolin can also exist in a cytosolic pool in complex with chaperones and cholesterol (57).

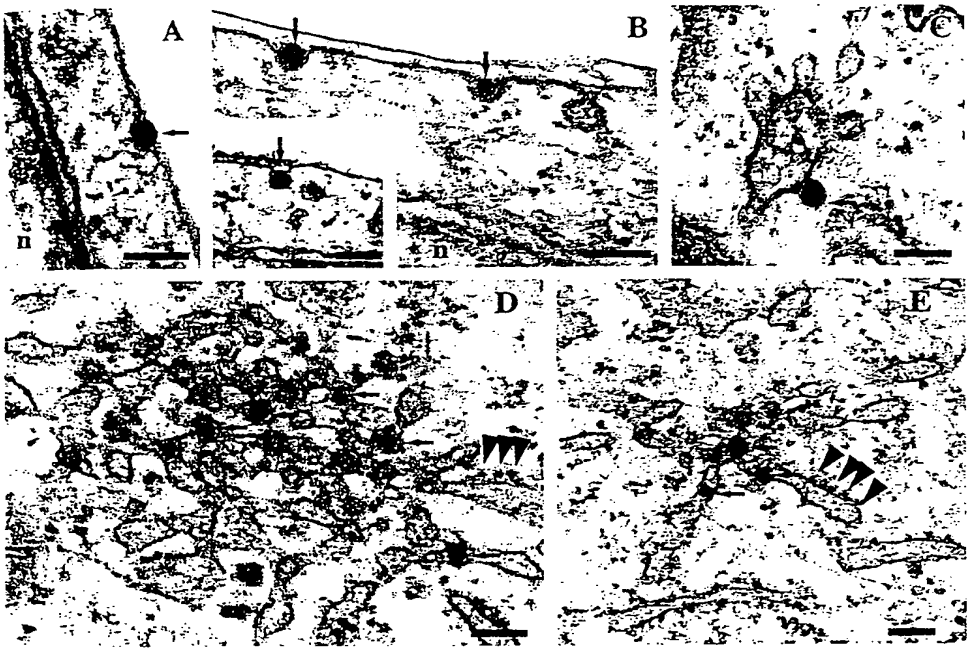

Fig. 1. Electron micrographs showing SV40 at different stages of the entry pathway. Vero cells were incubated with SV40 at 37°C for 3 h before fixation and processing for electron microscopy. Panels A–C show SV40 particles (arrows) at different stages of the invagination process at the plasma membrane. In A, the virus is in a shallow pit, in B these are deeper, and in the inset to B and in panel C the virus particles are almost completely enclosed by the enwrapping membrane. Note that the unoccupied caveolae indicated by 'x' in panels B and C are larger than those containing virus, suggesting that the caveolin-containing membranes may form around the virus particles. In C, a virus particle is evident in a multilobed surface-connected invagination. The multilobed appearance is very characteristic of caveolae in many different cell types (see (23)). Panels D and E show virus particles within the cell after a further 15 h incubation. The virus particles (small arrows) are in smooth surfaced tubules which are clearly connected to ribosome-studded rough ER (ribosomes indicated by arrowheads). n, nucleus. Bars, 100 nm.

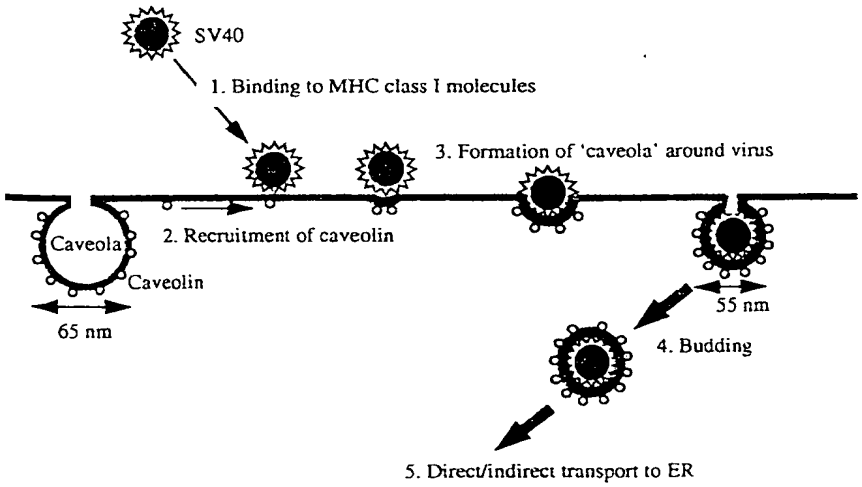

Fig. 2. Model for SV40 entry via caveolin-positive surface pits. In this hypothetical scheme, SV40 binding to MHC class I causes caveolin to be recruited around the virus particle. This ultimately generates a virus-containing pit in which the membrane is very tightly apposed to the virus surface (see Fig. 1). This pit is distinct from existing caveolae, which are generally larger. The virus-containing pit eventually becomes separated from the membrane and, directly or indirectly, reaches the endoplasmic reticulum (ER). Note that for simplicity MHC class I and other potential receptors are not shown in the scheme. At some stage in the entry pathway the virus must exit a membrane-bound compartment and reach the cytosol.

Bacterial uptake: analogous mechanisms

The recruitment of caveolin around the virus particle during the infectious entry process could sequester caveolin-associated cellular machinery onto the cytoplasmic face of the underlying membrane. This may set in motion a series of events, including signal transduction (55), which results in the internalization and further transport of the virus. Recent studies (58) raise the possibility that a similar process might have been exploited by a completely different pathogen. While most E. coli strains survive extracellularly, a uropathogenic E.coli strain enters macrophages and escapes degradation. This property relies on the interaction between the E. coli FimH protein and a GPI-anchored protein, CD48 (58). There are some striking parallels to the SV40 entry pathway. Firstly, the entry process is specifically blocked by cholesterol-disrupting agents. Second, and most interestingly, caveolin-1 is recruited to the enclosing membrane. This process is dependent on the interaction of the FimH with the GPI-anchored CD48 and results in a closely fitting enclosing membrane which apparently 'zips' up around the bacterium. The FimH-positive bacteria utilizing this pathway enter a non-acidic compartment and escape degradation, whereas opsonized bacteria, phagocytosed in a conventional fashion, are rapidly targeted to phagolysosomes and degraded (58). Together, these results raise the interesting possibility that the clustering of the GPI-anchored protein, which has affinity for glycosphingolipid-enriched DIG domains (21), is able to recruit caveolin and so initiate a chain of events leading to specific entry of the bacterium into a non-degradative pathway, analogous to that described above. While the nature of the intracellular compartment containing the FimH bacteria is unclear, it is interesting to note that other bacteria, such as *Brucella*, have exploited the ER as a protected intracellular niche in which replication can occur (59).

Surface to ER transport: comparisons and speculations

After internalization via the caveolin-enriched uncoated pits described above, SV40 particles accumulate in the ER (8). The molecular characterization of this compartment may give clues to the process by which the virus reaches the cytosol. The pathway by which the virus reaches the ER is of great interest to cell biologists as well as apparently being vital to understanding the infection process. In one possible scenario the virus-containing structures fuse directly with the ER. This would be consistent with the majority of ultrastructural studies, which failed to observe obvious intermediates in the entry pathway (e.g. (8)). However, SV40 entry is highly asynchronous, and so these transient intermediates may not be readily visualized. An alternativ scenario involves the transient transport of virus particle through endosomes and then the Golgi complex. A number c studies from other systems have suggested that budded caveola fuse with early endosomes in cultured endothelial cells and i fibroblasts (17, 42). This is consistent with the observation th SV40 particles have been observed in endosomes. In many case this was interpreted as misrouted virus destined for degrad tion, and this is consistent with studies with lysosomotrop agents, which showed that these agents did not inhibit SV4 infection (60). It is also possible that transport to the ER occu via the Golgi complex rather than directly to the ER.

In view of the lack of convincing experimental data f SV40 routing, it is of interest to examine the transport of oth ligands which pass from the surface to the ER. It is particular interesting to note that several ligands are apparently tran ported to the ER from surface caveolae. We will briefly consid two such ligands which may follow this route, cholera tox and autocrine motility factor (AMF). Cholera toxin routin represents possibly the best-characterized retrograde transpc pathway from the cell surface to the ER. Each cholera tox molecule comprises five chains involved in binding the toxin surface GM1 and one active chain with enzymatic activi Cholera toxin binds to surface GM1 present over the entire su face but is concentrated in caveolae, this concentration bei promoted by the multivalent nature of the ligand (2 Although cholera toxin is also present in coated pits, it appe that it is the toxin present in caveolae or caveolae-like D domains (in cells lacking morphologically identifiable cave lae) which leads to toxicity (61, 62). This is an intrigui observation because the active subunit of the toxin must p all the way to the ER to have a toxic effect; this pathway involv transport to endosomes and then the Golgi complex (63) a yet the activity of the toxin is dependent on the initial bindi event in caveolae-like domains. This was shown by eleg experiments utilizing hybrids of cholera toxin and a clos related toxin, heat labile toxin, which differs from chol toxin in its lipid-binding specificity (61). Upon reaching t Golgi complex, the binding and active subunits of chol toxin are dissociated and the active subunit is transported to t ER in a retrograde transport pathway utilizing a KDEL sig and the host cell KDEL-receptor retrieval system (64). T pathway shows similarities to that followed by SV40 as it blocked by cholesterol-disrupting agents and by brefeldin (65) (E. Stang, M. Lindsay, N. Zorzi, R.G. Parton, unpublish results). However, at present no other evidence exists for trar through the Golgi complex in the SV40 entry pathway.

Another molecule, which apparently follows a caveola to ER pathway, is AMF, a secreted growth factor (66). Recent studies showed that AMF binds to its receptor on the plasma membrane and is initially detectable in caveolae (67). It then passes to an intracellular tubular compartment which is smooth surfaced but apparently continuous with the rough ER (68). This pathway is remarkably similar to that followed by SV40, although the kinetics of virus entry appear much slower. Like AMF, SV40 accumulates in a specialized domain of the ER. The compartment in which SV40 accumulates comprises smooth-surfaced tubules which are connected to the ribosome-studded rough ER (see Figs 1D &1E) and are positive for protein disulphide isomerase, but not for ER/Golgi intermediate compartment markers (E. Stang, R.G. Parton, unpublished results). Whether the compartments are identical and, if so, why a virus and a ligand-receptor complex should be delivered to this compartment is unclear. Possibly some specialized function of this compartment is required for virus translocation and for late events in AMF signal transduction. It is also possible that this trafficking pathway is linked to the cycling of caveolin between surface caveolae and the ER, as outlined above. Whatever the function of this compartment and trafficking pathway, the further comparison of the AMF and SV40 trafficking routes with that of caveolin should prove illuminating.

Conclusions

Numerous basic questions, which are crucial to understanding the infectious entry of SV40, still remain. Most importantly, the compartment from which the virus reaches the cytosol is unclear. The high resolution structural analysis of SV40 and the related polyoma virus will undoubtedly provide more clues into the actual translocation process (69, 70). Meanwhile, the application of modern cell biological techniques to the trafficking of the virus should provide further clues as to the molecular machinery utilized by the virus to reach its site of membrane translocation. In view of the importance of the association of other retrogradely transported molecules, such as cholera toxin, with DIG/caveolae domains at the cell surface, this will provide an important avenue of future research into this fascinating pathway.

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DNA-Binding Properties of the Major Structural Protein of Simian Virus 40

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We investigated whether the VP1 protein of simian virus 40 binds to DNA. In vitro DNA-binding experiments clearly indicate that VP1 bound strongly to double-stranded and single-stranded DNA, with a higher affinity for the latter; additional experiments show that VP1 did not bind to a specific sequence of simian virus 40 DNA.

Alth ugh the DNA-binding properties of simian virus 40 (SV40) large T antigen (T-Ag) are well characterized, no such properties have been described for the SV40 capsid proteins. Nevertheless, several studies have shown that dissociation of mature virions in vitro leads to the formation of a nucleoprotein complex containing the viral DNA and a fraction of the viral capsid proteins VP1 and VP2. Treatment of this complex with high-salt (4) or detergent (6) solutions has shown that the VP1-DNA interaction is rather stable. These observations led us to examine directly the DNA-binding properties of VP1.

Confluent monolayers of CV1 cells were infected with SV40 at a multiplicity of infection of 10 PFU per cell. Twenty-one hours after infection, the cells were labeled for 3 h with 300 μCi of [^{35}S]methionine in 2 ml of methionine-free medium per 10-cm petri dish. After labeling, nuclear proteins were extracted as described by May et al. (9) with certain modifications. Briefly, the cells were washed with phosphate-buffered saline and incubated in 0.5 ml of 0.1% Tween 80 per petri dish for 5 min. Ice-cold isotonic buffer (10 mM triethanolamine [pH 7.0], 0.25 M sucrose, 25 mM NaCl, 1 mM EDTA) was then added (3 ml per petri dish). The nuclei were pelleted at 1,500 × *g* for 5 min, washed with isotonic buffer three times, and lysed in 1.0 M NaCl (1 ml/5 petri dishes) for 30 min at 4°C. The suspension obtained was centrifuged at 20,000 × *g* for 60 min, and the supernatant was dialyzed overnight against column buffer (10 mM sodium phosphate [pH 7.4], 0.1 M NaCl, 10% glycerol [wt/vol], 0.5% Nonidet P-40, 1 mM EDTA) and clarified by centrifugation at 8,000 × *g* for 15 min. Such extracts were stored at −70°C. All buffers contained 1 mM phenylmethylsulfonyl fluoride and 1 μg of leupeptine per ml.

A nuclear extract prepared from SV40-infected cells as described above was passed through a double-stranded DNA (dsDNA)-Sephadex column (calf thymus DNA) (5), and bound proteins were then eluted with column buffer of increasing ionic strengh. The collected fractions were immunoprecipitated with either Hamster tumor serum or anti-SV40 capsid serum and then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Fig. 1A shows the result of immunoprecipitation of the extract before applying it to the column. At 24 h after infection, substantial quantities of VP1 protein were found in the nucleus. This is consistent with earlier findings that viral capsid proteins are transported to the nucleus as early as 13 h after infection (2, 16). As expected, there were also large amounts of T-Ag. Analysis of the eluted fractions shows that, under our loading conditions (pH 7.4), a large portion of this T-Ag did not bind to the DNA. The bound fraction was eluted at 0.2 M NaCl (Fig. 1B). This is in good agreement with previous results described by others (13). We found that a certain fraction of VP1 bound strongly to the column under these conditions. It was eluted at 0.35 M NaCl and thus bound to the DNA more strongly than did T-Ag (Fig. 1). However, there was also a fraction of VP1 which did not bind to the column under these conditions (pH 7.4, 0.1 M NaCl). This nonbinding fraction of VP1 was consistently seen in several independent experiments. To verify that this was not due to saturation of the column, the 0.1, 0.2, and 0.35 M NaCl fractions were rechromatographed on dsDNA-Sephadex columns. Each fraction displayed an unchanged affinity to DNA (data not shown). It is known that a minor fraction of VP1 is phosphorylated (12). Under our experimental conditions this form of VP1 represented a very s

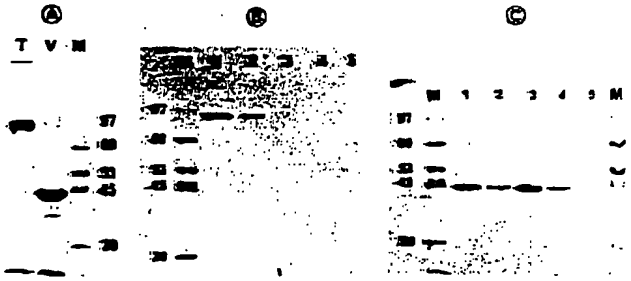

FIG. 1. Chromatographic behavior of T-Ag and VP1 on a dsDNA-Sephadex column. (A) Nuclear extract of SV40-infected cells immunoprecipitated with hamster tumor serum (lane T) or anti-SV40 capsid serum (lane V). (B and C) A 0.2-ml portion of extract in column buffer was loaded onto a 1-ml dsDNA-Sephadex column (approximately 0.5 mg of DNA). The column was washed, and then proteins were eluted with buffers of increasing ionic strength, and the eluted fractions were immunoprecipitated with hamster tumor serum (B) or anti-SV40 capsid serum (C). Immunoprecipitated proteins were then analyzed by electrophoresis through an SDS–10% polyacrylamide gel, followed by fluorography of the gels. The salt concentrations of NaCl in the loading and elution buffers were 0.1 M (lane 1 [flow through]), 0.2 M NaCl (lane 2), 0.35 M (lane 3), 0.5 M (lane 4), and 1.0 M (lane 5). Lanes M contain molecular weight markers (molecular weights are indicated in thousands next to lanes).

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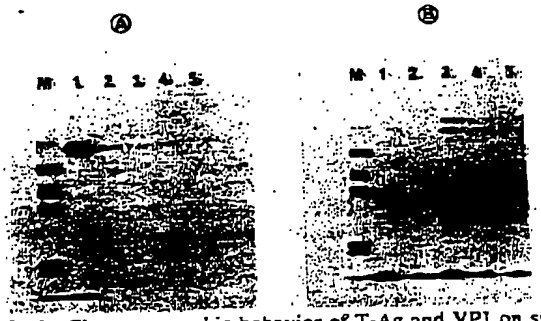

FIG. 2. Chromatographic behavior of T-Ag and VP1 on ssDNA ultrogel column. Procedures and lane designations are described in the legend to Fig. 1. (A) Eluates immunoprecipitated with hamster tumor serum. (B) Eluates immunoprecipitated with anti-SV40 capsid serum.

portion of the total VP1 population, too small to correspond to either the binding or nonbinding forms observed here (data not shown). Since VP1 contains seven cysteine residues and intermolecular disulphide bonding is known to be involved in capsid formation, it was possible that the unbound VP1 might represent aggregates of VP1 incompletely dissociated by the extraction procedure used. We therefore repeated the experiment, incubating the nuclear extract in 0.5 M β-mercaptoethanol for 30 min at 4°C to ensure the complete dissociation of all complexes. However, the results obtained (data not shown) were the same as those shown in Fig. 1C. Thus, under all conditions used, we consistently found a subpopulation of VP1 which did not bind to dsDNA. At present we cannot say whether this represents VP1 which has lost its affinity for dsDNA during the extraction procedure or whether it represents a distinct population devoid of this function.

We also examined the binding of VP1 to single-stranded DNA (ssDNA)-ultrogel column (Fig. 2B). Again, some VP1 did not bind to ssDNA. A peak of bound VP1 was eluted at 0.35 M NaCl, but a substantial portion of VP1 was not eluted until 0.5 or 1.0 M NaCl. T-Ag did not bind significantly to ssDNA under these conditions (Fig. 2A).

To verify that VP1 interacts directly with DNA (and not via another protein) we tested the binding of VP1 to dsDNA by using the Western blot technique. Nuclear extracts from infected CV1 cells were immunoprecipitated with anti-VP1 serum, separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose filter, and incubated with labeled SV40 DNA. Only one band was visible, corresponding to a protein with a molecular weight of 45,000 (Fig. 3, lane 2). When similar experiments were carried out with uninfected CV1 cells (Fig. 3, lane 1) or Cos cells (lane 3), both of which lack VP1, no bands were visible. It thus appears likely that VP1 interacts directly with DNA.

We used the McKay binding assay (10) to examine whether VP1 binds to a specific sequence of SV40 DNA. SV40 DNA was double digested with *Hin*dIII and *Hpa*II and end-labeled by using Klenow DNA polymerase and ^{32}P-labeled dATP. The conditions of binding and immunoprecipitation have already been described (7). Immunoprecipitation was performed with anti-tumor serum (Fig. 4, lane T) and anti-VP1 serum (lanes 1 through 6). Under these conditions (0.2 M NaCl; pH 7.4), even in the presence of an excess of calf thymus DNA, T-Ag bound strongly and specifically to fragment F, which contains the origin of DNA replication. In the absence of calf thymus DNA, VP1 bound equally to all

FIG. 3. Analysis of the binding of VP1 to DNA by the Western blot procedure. Nuclear extracts from uninfected CV1 cells (lane 1), infected CV1 cells (lane 2), or Cos cells (lane 3) were immunoprecipitated with anti-VP1 serum and separated by SDS-polyacrylamide gel electropohoresis. The proteins were transferred to a nitrocellulose filter as described previously (8). Prebinding was carried out for 3 h at 37°C in a buffer containing 10 mM sodium phosphate (pH 7.4), 1% (wt/vol) glycerol, 0.5% Nonidet P-40, 0.2 M NaCl, and 3% ultrapure bovine serum albumin. Binding with 50 ng of SV40 DNA labeled by nick translation (15) was performed in the same buffer for 16 h at room temperature with gentle agitation. The filter was washed four times for 15 min each at room temperature in the same buffer without bovine serum albumin.

fragments of SV40 DNA (Fig. 4, lane 1). Addition of increasing quantities of calf thymus DNA led to a parallel loss of binding to all fragments (Fig. 4, lanes 2 through 6). This indicates the absence of a strong affinity site for VP1 binding to SV40 DNA. However, with this technique we cannot exclude the presence of a specific low-affinity site.

The data presented in this communication clearly show that (i) VP1 had a high affinity for DNA, (ii) this affinity was higher for ssDNA than for dsDNA, (iii) the affinity of VP1 for dsDNA was higher than that of T-Ag, and (iv) VP1 interacted directly with the DNA but did not bind specifi-

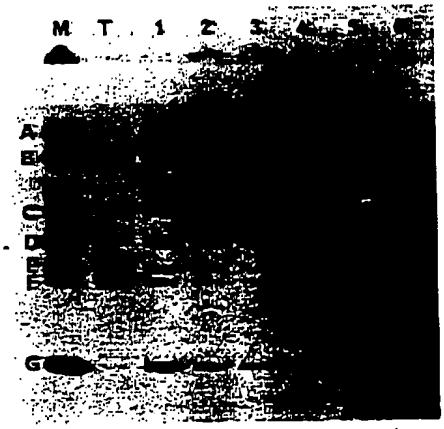

FIG. 4. Binding of T-Ag and VP1 to SV40 DNA. End-labeled SV40 DNA (100 ng) was incubated with 10 μl of nuclear extract from infected CV1 cells in a buffer containing 10 mM sodium phosphate (pH 7.4), 200 mM NaCl, 10% (wt/vol) glycerol, 0.5% Nonidet P-40, 1 mM EDTA, and the following quantities of sheared calf thymus DNA: 5 μg (lanes T and 6), none (lane 1), 0.5 μg (lane 2), 1 μg (lane 3), 2 μg (lane 4), and 3 μg (lane 5). Immunoprecipitation and electrophoresis were performed as described previously (7).

cally to any particular site on SV40 dsDNA. This interaction might reflect an important role for VP1 during SV40 morphogenesis. It has already been demonstrated that SV40 DNA is stably associated with capsid proteins (4, 6). This association is probably a consequence of the DNA-binding capacity of VP1 demonstrated here. Our finding supports the model of morphogenesis in which the capsid is organized by the gradual addition of capsid proteins to SV40 chromatin (3). A similar situation appears to exist in the case of the hepatitis B virus (HBV) since it has recently been shown by Petit and Pillot (14) that the major core protein p22 of HBV is a DNA-binding protein. Furthermore, the affinity of p22 is higher for HBV DNA than for plasmid DNA, and Petit and Pillot also suggest a role for p22 in HBV assembly.

Another possible role for the binding of VP1 to DNA has been suggested by Brady et al. (6), who have shown that VP1 (or VP2) increases transcription from the SV40 minichromosome in vitro. This stimulation is correlatd with the association of VP1 to the minichromosome. A potential mechanism for the stimulation of transcription is suggested by Moyne et al. (11), who propose that VP1 can act as a nucleosome-unfolding agent. It is possible that the unfolding of nucleosomes on the SV40 minichromosome facilitates viral transcription or replication or both. We can postulate that the DNA-binding capacity of VP1 is associated with its nucleosome-unfolding activity. However our results suggest that this is not due to the binding of VP1 to any specific site on SV40 DNA.

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Identification of a DNA Binding Domain in Simian Virus 40 Capsid Proteins Vp2 and Vp3*

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We have identified both biochemically and genetically a protein domain within the simian virus 40 virion protein Vp3, and within Vp2 since its carboxyl two-thirds are identical to the full-length Vp3, that binds DNA in a sequence nonspecific manner. Both the Vp2 and Vp3 (Vp2/3) components of SV40 and mutant $SV40_{202T}$ bound either SV40 or pBR322 DNA equally well. Wild type and mutant Vp2/3 proteins, expressed as fusion proteins with glutathione *S*-transferase (GST), were tested for their ability to bind DNA. GST-Vp3 bound DNA at physiological salt concentrations with an apparent K_d of 2.5×10^{-8} M and also bound RNA with 4-fold higher affinity. Over 90% of the nucleic acid binding, and all of the activity, was lost upon removal of the carboxyl-terminal 13 and 35 residues, respectively. The DNA binding domain was shown to be distinct and separable from the Vp2/3 nuclear transport signal since mutations within the nuclear transport signal that reduce or abolish nuclear localization of Vp2/3 had no effect on the DNA binding activity of mutant Vp2/3 fusion proteins. The carboxyl-terminal 40 residues of Vp2/3 in the form of a β-galactosidase fusion protein, F6, are sufficient for DNA binding and may cause compaction of the DNA. The significance of this DNA binding and possible compaction are discussed in relation to the assembly of virion particles.

The papovaviruses, including murine polyoma virus and simian virus 40 (SV40),[1] are icosahedrally symmetric viruses whose capsids are composed of 72 pentamers of the major structural protein Vp1 (1–3). The mature virion also contains the viral-encoded proteins Vp2 and Vp3, and histones H2A, H2B, H3, and H4 (4). Based on the measurements of the molar ratio of Vp1, Vp2, and Vp3 in virions, there are about 21 Vp2 and 56 Vp3 molecules present in a particle (5). A reasonable assumption then would be that each of the 72 Vp1 pentamers interacts with either one Vp2 or one Vp3 molecule. Vp2 contains all of the amino acids of Vp3 plus an additional 118 amino-terminal residues and as such, the interaction of Vp2 and Vp3 with the Vp1 pentamers could be the same. The electron density map of polyoma virions at 25-Å resolution has shown the interior as a mine-shaped structure with 72 prongs, each of which has been proposed to be one molecule of either Vp2 or Vp3, each radiating from the central minichromosome core into the axial cavities of the Vp1 pentamers (6). The interior structure resolved by the x-ray diffraction data is in agreement with that obtained by electron cryotomy of SV40 particles (1). At the core of the mine-shaped structure, the Vp2 and Vp3 molecules are predicted to interact with the SV40 minichromosome, which consists of a viral, covalently closed circular DNA of 5243 bp and about 21 histone octamers (7–9). In housing the interior structure, the Vp1 pentamer is expected to interact with Vp2 and Vp3 as well as with the minichromosome. SV40 Vp1 has been shown to bind DNA (10), and a sequence nonspecific DNA binding domain in polyoma Vp1 also has been identified within its first 7 amino acids (11). Furthermore, a Vp1-interactive domain has been identified in Vp3 of SV40 (12).

Several lines of evidence suggest that Vp3 interacts with the viral minichromosome and DNA. In addition to its proximity to the viral DNA in the virion particle, Vp3 has been shown to remain complexed with the minichromosome upon dissociation of virions (13, 14). Histone H1, which is absent in the mature SV40, has been reported to be present in the nuclear form of the SV40 minichromosome (15, 16). Thus it has been suggested that one role of Vp3 is to replace H1 and condense the minichromosome during virion packaging (16). A protein domain(s) within SV40 Vp2/3 could function in DNA binding and compaction during virion formation, as has been suggested (13, 15, 17).

The carboxyl-terminal 40 residues (195–234 of Vp3) of SV40 Vp2/3 contain multiple domains involved in virion morphogenesis. The Vp2/3 nuclear transport signal (NTS) has been localized to 9 residues (198–206 of Vp3) and has been shown to be necessary and sufficient for nuclear targeting of both the Vp3 protein and non-nuclear carrier proteins (18, 19). Residues 222–234 of Vp3 specify a Vp1-interactive determinant of Vp2/3: fusion of the last 40 residues of Vp3 to β-galactosidase promotes association with Vp1, while deletion of the last 13 residues abolishes the interaction between Vp3 and Vp1 (12, 18). Additionally, the last 40 residues are rich in basic residues, and several stretches of amino acid sequence similarity to histones H2A, H2B, and H1 are found within the carboxyl portion of Vp2/3 (17). In this report we have examined the interaction of SV40 Vp3 with DNA.

MATERIALS AND METHODS

Plasmid Constructions—Plasmids coding for glutathione *S*-transferase (GST)-Vp3 fusion proteins were constructed by subcloning 0.95 kilobase (kb) *Eco*47III to *Eco*RI restriction fragments of SV40 DNA (SV40 nucleotides 832–1782) into the *Sma*I/*Eco*RI-digested plasmid pGEX-3X, which contains the coding sequence for GST from

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[1] The abbreviations used are: SV40, simian virus 40; NTS, nuclear transport signal; GST, glutathione *S*-transferase; DTT, dithiothreitol; bp, base pair(s); kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis.

EXHIBIT
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Schistosoma japonicum under control of the inducible *tac* promoter (20; Pharmacia LKB Biotechnology Inc.). References to SV40 nucleotide numbers are in the SV system (4). DNA manipulations were performed as described (21). All of the GST fusion proteins contain 27 unique Vp2 residues (Vp2 residues 92–118) upstream of the Vp3 initiation codon in addition to Vp3. For the construction of the Vp2/3 NTS mutant GST-Vp3 expression vectors pGEX-$Vp3_{202T}$, pGEX-$Vp3_{204T}$, pGEX-$Vp3_{202E}$, pGEX-$Vp3_{202E/204T}$, and pGEX-$Vp3_{202A/204T}$, we first constructed $pSV40_{202T}$, $pSV40_{204T}$, $pSV40_{202E}$, $pSV40_{202E/204T}$, and $pSV40_{202A/204T}$ from a mutant phage stock (19) and their *Eco*47III to *Eco*RI fragments were used as described above. Briefly, the *Kpn*I-*Eco*RI (SV40 nucleotide 294–1782) fragments in M13 mp19 containing the point mutations were exchanged with their wild type counterparts from the Vp2/3 transfection vector, pSVP23A (12). The *Eco*RI fragment from pSp6Vp1 (12) which has the Vp1 coding sequence and SV40 sequence up to 2666 was inserted into the resulting mutant pSVp23A plasmids to yield the mutant pSV40 vectors.

The plasmid pGEX-Vp3ΔC35 was created by subcloning the *Eco*47III to the *Eco*RI fragment derived from the plasmid pSVP23AΔC35 (17) into pGEX-3X. The plasmid pGEX-Vp3ΔC13 was made by replacing the *Avr*II (SV40 nucleotide 1078) to *Eco*RI restriction fragment from pGEX-$Vp3_{204T}$ with its counterpart fragment (*Avr*II to *Eco*RI) derived from the plasmid Sp6Vp3ΔC13 (18). In the mutant Vp3ΔC35, the carboxyl-terminal 35 residues of Vp3 were deleted and the protein contained an additional 3 residues (LTD) at the end. In the mutant Vp3ΔC13, the last 13 residues of Vp3 were deleted and the protein aquired 6 additional residues (LTDNSS) at the end.

The plasmids B290E1, B290F6, and $B290F6_{202T}$ have been described (12, 19). All constructions were verified by restriction enzyme analysis and by dideoxynucleotide, double-stranded, plasmid sequencing (22, 23), as well as by their ability to produce anti-Vp3 immunoreactive species with the expected molecular weights.

Antibodies and Immunoblotting—Affinity-purified anti-Vp3 IgG was prepared from polyclonal rabbit anti-Vp3 sera (24) using SV40 virion-derived Vp3 immobilized on nitrocellulose blots as described (25). Immunoblotting was performed as described (5).

Cells and Preparation of Virions—The conditions for cell culture of TC7 and MA134 cells, both sublines of African green monkey kidney cells, and virion propagation have been described (19). Wild type SV40 and $SV40_{202T}$ virions were purified 10 days postinfection from infected MA134 cell lysate supernatants by sequential KBr and CsCl gradient centrifugation as described (26).

An SV40 virus containing a point mutation, $SV40_{202T}$, was derived from the plasmid $pSV40_{202T}$ by digesting with *Bam*HI to separate the pBR322 and SV40 sequences and ligating with T4 ligase under conditions which promote intramolecular *versus* intermolecular ligation (21). The ligated $SV40_{202T}$ DNA was microinjected into TC7 cells which then were prepared for the plaque assay as described (27, 28). After 20 days, a plaque was isolated and used to infect cells to make a stock viral lysate from which infected cells were made to prepare mutant DNA and mutant virus. Viral DNA was extracted (29), CsCl banded, and double-strand sequenced to confirm the presence of the mutation.

Isolation of GST-Vp3 Fusion Proteins—*Escherichia coli* strain RRI (21) harboring the pGEX-Vp3 expression vectors was used to overexpress the GST-Vp3 fusion proteins. Cells, induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 2–4 h, were washed with 20 mM Hepes, pH 7.3, 50 mM KCl, and 10% glycerol (v/v), resuspended in 0.02 volumes of the same buffer, and disrupted by sonication on ice. The majority of fusion proteins (90–95%) were insoluble and were separated from the homogenate by centrifugation at 12,500 × *g* for 15 min. Those present in the soluble fraction were affinity-purified using a 3-ml cross-linked agarose-glutathione column (20). The eluate from the column was dialyzed to remove the free glutathione and the proteins were concentrated using Centricon-30 concentrators (Amicon) and stored in aliquots at −70 °C at 2 mg/ml (30). After affinity purification, only the fusion protein and GST were present in the eluate based upon Coomassie Brilliant Blue staining of SDS-polyacrylamide gels (data not shown).

The sonicate pellets were resuspended and washed at least 3 times with 10 mM Hepes, pH 7.3, 5 mM EDTA, and 1% Triton X-100 (v/v) to remove membrane proteins, and were dissolved in 8 M urea to 1/50 of the original culture volume. Solubilized proteins were renatured by dialysis without stirring for 16 h to achieve a final concentration of 0.5 M urea in 25 mM Tris-HCl, pH 7.5. This was dialyzed further with stirring against 25 mM Tris-HCl, pH 7.5, to reduce the concentration of urea to below 1 μM. The precipitate which formed upon dialysis was removed by centrifugation at 12,500 × *g* for 10 min. These solubilized proteins were analyzed by SDS-PAGE after which the amount of fusion proteins were determined, aliquoted, and stored at −70 °C at a concentration of 1–2 mg/ml. The fusion proteins constituted about 20–50% by mass of the total protein (Fig. 2*A*). Typically, 1 liter of induced cells yielded 5–10 mg of relatively stable Vp3 fusion protein. Both affinity-purified and urea-solubilized fusion proteins bound DNA equally well.

Fractionation of β-Galactosidase Fusion Proteins—β-Galactosidase-Vp3 fusion proteins were fractionated as described previously (19). The fusion proteins in the gel filtration eluate constituted at least 90% of the protein mass, judging from SDS-PAGE and Coomassie Blue staining and from immunoblots using monoclonal anti-β-galactosidase IgG.

Radiolabeling of DNA and RNA—DNAs were nick-translated using [α-^{32}P]dATP (specific activity: 3000 Ci/mmol; ICN, Irvine, CA) or 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (specific activity: 3000 Ci/mmol; ICN) as described (21). The 179- and 1541-bp fragments were derived from pSp6Vp1 (12) and the 476-, 1014-, and 5243-bp fragments were derived from SV40 DNA.

Radiolabeled RNAs were prepared by *in vitro* transcription using SP6 polymerase and [α-^{32}P]GTP (specific activity: 400 Ci/mmol; Amersham) as described (21). The transcription vectors pSp6Vp1 and pSp6Vp3 (12) were linearized with *Sst*I to make SV40-derived transcripts and pSP64 (31) was linearized with *Dde*I to generate a transcript of comparable size. After RNA synthesis, the DNA was digested with DNase I, and unincorporated nucleotides were removed by ethanol precipitation in the presence of ammonium acetate (21).

Detection of DNA Binding Proteins on Nitrocellulose Blots—For Southwestern assays, after SDS-PAGE (32) and electrotransfer of proteins to nitrocellulose, replicas were blocked in buffer A (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 6.7) containing 1 × Denhardt solution for at least 30 min at 22 °C (33). Nick-translated, ^{32}P-labeled pBR322 or SV40 DNAs (specific activity: 10^7 cpm/μg) were incubated with blots in sealed bags in the above buffer (10 ng/ml in 5 ml) with or without 10 mM DTT for 1 h at 22 °C. The blots were then washed twice for 5 min each in 50 ml of buffer A, twice for 15 min in 100 ml, and twice for 30 min in 100 ml, at 22 °C before being air-dried and exposed to film for autoradiography.

For nondenaturing dot blots, protein samples (0.1 μg) in 0.1 ml of 25 mM Tris-HCl, pH 7.5, were layered directly onto nitrocellulose by incubation overnight at 37 °C in a 96-well dot blot manifold. These blots were blocked, reacted with ^{32}P-labeled DNA, and washed exactly as described above. To determine the effects of salt on DNA binding, increasing concentrations of NaCl were added to both the incubation and wash buffers.

Gel Shift DNA Binding Assays—Varying amounts of proteins were incubated with 2 ng of ^{32}P-end-labeled DNA fragments (5000 cpm) for 30 min at 22 °C in 15 μl of buffer B (20 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT) containing 50 mM NaCl. After adding one-sixth volume of loading dye (0.04% bromphenol blue, 0.04% xylene cyanol, and 0.05% glycerol) the reaction mixtures were immediately electrophoresed on 6% polyacrylamide gels in TBE buffer (21) for 1–2 h at 20 mA. The gels were fixed for 30 min in 10% acetic acid, dried, and exposed for autoradiography. Alternatively after the 30-min incubation, proteinase K (30 μg) or SDS (3% final concentration) was added to the reaction mixtures which were then incubated for an additional 30 min at 37 °C prior to electrophoresis. For competition experiments, 0.1 or 10 μg of sheared salmon sperm or 0.15 μg of sheared *E. coli* DNA was added to the binding reactions.

Filter Binding Assays—Filter binding assays were performed in duplicate using affinity-purified GST-Vp3 and radiolabeled probe (1 ng; specific activity: 5×10^7 cpm/μg) in buffer A as described (34). For competition experiments, the indicated amounts of competitor nucleic acids were added to buffer A containing 0.16 μM GST-Vp3 immediately before addition of the radioactive probe. Background binding of the probe to the filters was corrected for by performing identical reactions in the absence of GST-Vp3 and typically accounted for less than 8% of the maximum counts bound. Nonradioactive SP64, Vp1, and Vp3 RNAs were prepared by *in vitro* transcription using *Dde*I-linearized pSP64, and *Sst*I-linearized pSp6Vp1 and pSp6Vp3, and quantitated spectrophotometrically as described (21). Single-stranded M13 mp18 DNA and yeast tRNA were from Life Technologies Inc.-Bethesda Research Laboratories.

Densitometry—Densitometry was performed on autoradiograms, within the linear response range of the film, using a Bio-Rad model 620 video densitometer.

RESULTS

DNA Binding by Virion-associated Vp3

We have purified wild type virions, as well as mutant virions containing a single amino acid change in the Vp2- and Vp3-NTS, Vp3 lysine 202 to threonine ($SV40_{202T}$), and tested their proteins (Fig. 1*A*, *lanes 2* and *3*) for their ability to bind nick-translated ^{32}P-labeled SV40 DNA in a Southwestern assay (Fig. 1*B*). All of the virion proteins, Vp1, Vp2, and Vp3 of both wild type (Fig. 1*B*, *lane 2*) and $SV40_{202T}$ (Fig. 1*B*, *lane 3*) bound DNA, while non-virion proteins, *e.g.* molecular weight markers, did not (Fig. 1*B*, *lane 1*). Virion-associated histones also bound DNA (Fig. 1*B*). The source of labeled DNA did not appear to affect binding. Nick-translated pBR322 plasmid DNA was also used and gave the same results as SV40 DNA (data not shown). These results suggest that Vp1, Vp2, and Vp3 contain DNA binding domains and that the lysine at residue 202 of Vp3 (and the corresponding residue of Vp2, referred to collectively as Vp2/3) was not essential for this binding activity. Bacterially expressed polyomavirus Vp1 has been shown to bind DNA, but only when DTT is absent from the binding buffer (11). We tested if this was also the case for the SV40 protein, and found that the presence or absence of DTT did not effect the level of DNA binding by any of the SV40 proteins (data not shown).

DNA Binding by GST-Vp3 Fusion Proteins

Southwestern Analysis—In order to identify the domain(s) of Vp3 responsible for this Vp3 DNA binding activity, full-length Vp3 and two Vp3 carboxyl-terminal deletions were expressed as fusion proteins with GST. The fusion proteins, from the insoluble fraction of lysed cells, were separated by SDS-PAGE, visualized by Coomassie Blue staining (Fig. 2*A*), and transferred to nitrocellulose (Fig. 2, *B* and *C*). When reacted with affinity-purified rabbit anti-Vp3 IgG followed by ^{125}I-labeled protein A (Fig. 2*B*), one major and four minor anti-Vp3 reacting bands were present (Fig. 2*B*, *lane 1*). The major anti-Vp3 reacting band corresponded to the major band seen by mass (compare Fig. 2, *B*, *lane 1*, with *A*, *lane 1*), and corresponds to the expected size of the GST-Vp3 (53–55 kDa). The source of the minor immunoreactive bands is unknown

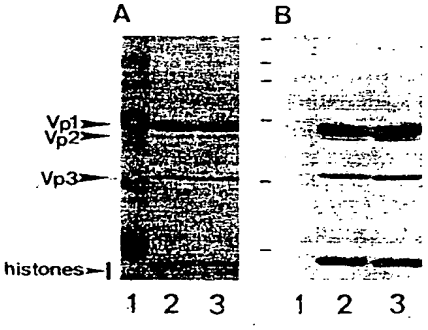

FIG. 1. **SV40 virion proteins bind DNA.** Wild type or $SV40_{202T}$ mutant virions were isolated and separated on 12.5% SDS-polyacrylamide gels before being Coomassie Blue stained (*A*) or transferred to nitrocellulose and probed with ^{32}P-labeled SV40 DNA (10^5 cpm/ml) in buffer A containing 1 × Denhardt solution and 10 mM DTT (*B*). Five μg of total viral proteins were loaded per lane in *A* while 10 μg were loaded per lane in *B*. *Lanes 2* (*A* and *B*) contain SV40 and *lanes 3* (*A* and *B*) contain $SV40_{202T}$ virions. The positions of Vp1, Vp2, Vp3, and histones are indicated to the *left* of *A* while the relative positions of the molecular weight markers (*lane 1*) are indicated as *bars* to the *left* of *B*. The molecular weight standards, from top to bottom, are myosin (H-chain) (200 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and β-lactoglobulin (18.4 kDa).

but we believe that the faster migrating bands are degraded fusion proteins.

When the proteins, after transfer to nitrocellulose, were incubated with ^{32}P-labeled DNA, the wild type GST-Vp3 bound the labeled DNA (Fig. 2*C*, *lane 1*). The major immunoreactive band as well as 2 of the faster migrating GST-Vp3 related bands bound DNA, while none of the other proteins present on the blot reacted with the DNA. DNA binding by GST-Vp3 was abolished in 0.3 M NaCl, but was still detectable in 0.2 M NaCl and was strongest in 0.05 M NaCl (data not shown).

To localize the Vp3 DNA binding domain, we constructed two carboxyl-terminal deletion mutants of Vp3, since the last 40 residues show a high degree of sequence similarity to regions of histones H1, H2A, and H2B (17). Two deletion mutant fusion proteins which removed either the last 13 (GST-Vp3ΔC13; Fig. 2, *A* and *B*, *lane 2*) or 35 (GST-Vp3ΔC35; Fig. 2, *A* and *B*, *lane 3*) residues of Vp3 failed to bind DNA by this assay (Fig. 2*C*, *lanes 2* and *3*, respectively). However, residual DNA binding activity of GST-Vp3ΔC13 was demonstrated with protein samples that had not undergone SDS denaturation. Using a dot blot assay, GST-Vp3 reproducibly bound DNA at NaCl concentrations up to 0.3 M (Fig. 2*D*, *1*; data not shown). While GST-Vp3ΔC35 failed to bind DNA above background levels (Fig. 2*D*, *3*), faint DNA binding by GST-Vp3ΔC13 was detected (Fig. 2*D*, *2*). Densitometry of autoradiograms revealed that GST-Vp3ΔC13 bound 6.7 ± 0.3% of wild type GST-Vp3 levels, based on data from three experiments (data not shown).

Separation of the Vp3 DNA Binding Domain and the Nuclear-Transport Signal—Since the mutant $Vp3_{202T}$ bound DNA to the same extent as the wild type Vp3 (Fig. 1), the Vp2/3-NTS (residues 198–206: GPNKKKRKL), which is highly basic and includes the 202 lysine, appears not to be a part of the DNA binding domain. To confirm that the NTS and the Vp3 DNA binding domain are independent of each other, we tested the DNA binding activity of 5 mutant proteins, whose single or double point mutations are within the Vp2/3-NTS. These mutations in GST-$Vp3_{202T}$, GST-$Vp3_{204T}$, GST-$Vp3_{202E}$, GST-$Vp3_{202E/204T}$, and GST-$Vp3_{202A/204T}$, change either Vp3 lysine 202 and/or arginine 204, both of which affect the function of this nuclear targeting domain (19, 44). All 5 mutant proteins reacted with anti-Vp3 IgG (Fig. 2*B*, *lanes 4–8*) and bound ^{32}P-labeled DNA by Southwestern and dot blot assays (Fig. 2*C*, *lanes 4–8* and *D*, *lanes 4–8*). These results indicate that the Vp2/3-NTS is functionally independent of the DNA binding domain, since mutations which are deleterious to Vp3 nuclear translocation did not greatly affect its DNA binding ability.

DNA Binding by GST-Vp3 Fusion Proteins in Solution—Since the GST-Vp3 fusion proteins appeared to bind more favorably with DNA when assayed under nondenaturing conditions (Fig. 2*D*), we examined the interaction of DNA with the fusion protein in solution using a DNA fragment mobility shift assay. Fig. 3 shows the result of a typical experiment. When affinity-purified GST-Vp3 was added in increasing amounts to a 179-bp end-labeled DNA fragment, the protein-free DNA band (Fig. 3*A*, *lane 1*, *arrowhead*) disappeared and slower migrating bands appeared (Fig. 3*A*, *lanes 2* and *3*). As was seen with the Southwestern assays, neither of the truncated proteins, GST-Vp3ΔC13 or GST-Vp3ΔC35, bound the DNA fragment, and the protein-free DNA remained at its position with equivalent band intensity (Fig. 3*A*, *lanes 4–7*). The mobility shift assay was also performed with a longer DNA fragment (1541 bp). The results were similar to those for the 179-bp fragment: GST-Vp3 was able to retard the

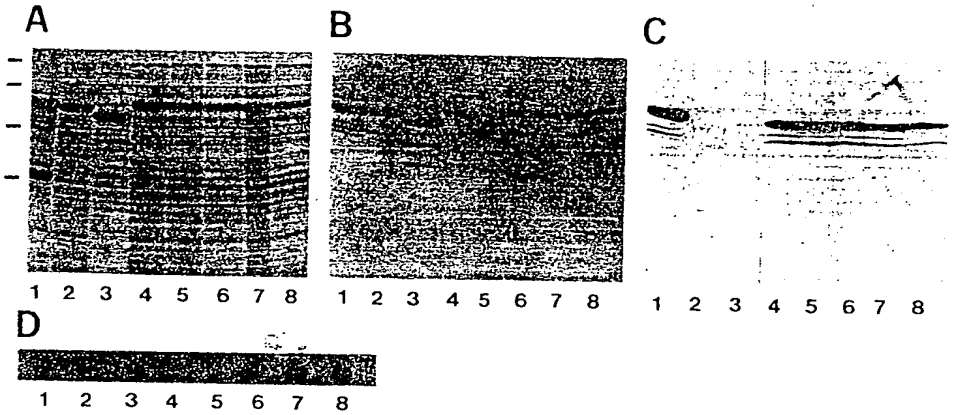

FIG. 2. **Western and Southwestern analyses of GST-Vp3 fusion proteins.** Approximately 4 μg of each of the urea-solubilized GST-Vp3 fusion proteins (8–15 μg of total protein) were separated per lane on 12.5% SDS-polyacrylamide gels and Coomassie Blue stained (*A*), or transferred to nitrocellulose and probed with 0.4 μg/ml affinity-purified anti-Vp3 IgG followed by ^{125}I-labeled protein A (*B*), or probed with nick-translated ^{32}P-labeled DNA in buffer A containing 1 × Denhardt solution without DTT (*C*). For dot blot analysis (*D*), 0.1 μg of each of the fusion proteins was blotted directly onto nitrocellulose and probed with ^{32}P-labeled DNA as in *C*. The samples are: GST-Vp3 (*lane 1*), GST-Vp3ΔC13 (*lane 2*), GST-Vp3ΔC35 (*lane 3*), GST-Vp3$_{202T}$ (*lane 4*), GST-Vp3$_{204T}$ (*lane 5*), GST-Vp3$_{202E}$ (*lane 6*), GST-Vp3$_{202E/204T}$ (*lane 7*), and GST-Vp3$_{202A/204T}$ (*lane 8*). Positions of molecular weight standards, phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa) are indicated, from the top to the bottom, respectively, to the *left* of *A*.

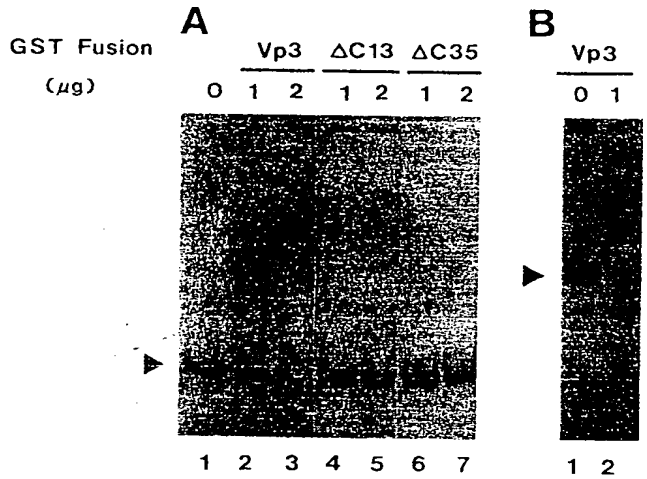

FIG. 3. **Binding of GST-Vp3 fusion proteins to DNA in solution.** *A*, affinity-purified GST-Vp3 (*lanes 2* and *3*), GST-Vp3ΔC13 (*lanes 4* and *5*), and GST-Vp3ΔC35 (*lanes 6* and *7*) were incubated with a 179-bp ^{32}P-end-labeled DNA fragment and separated by gel electrophoresis as described under "Materials and Methods." The amount of fusion protein added to each reaction is indicated at the *top* of the figure. DNA in the absence of protein is shown in *lane 1*. *B*, a 1541-bp ^{32}P-end-labeled DNA fragment was incubated in the absence (*lane 1*) or presence (*lane 2*) of GST-Vp3.

DNA fragment (Fig. 3*B*, compare *lanes 1* and *2*).

The addition of a 15- or 1500-fold molar excess of unlabeled sheared salmon sperm DNA to the reaction mixture together with the 179-bp ^{32}P-labeled DNA fragment abolished the mobility shift (data not shown). Thus the interaction of GST-Vp3 with DNA is sequence nonspecific, confirming the result presented above.

The mobility shift remained unaffected until the salt concentration was raised to 0.5 M and could still be detected at 1.25 M NaCl but not at 1.5 M NaCl (data not shown). That the interaction between GST-Vp3 and DNA is relatively stable at high salt concentrations was confirmed by independent experiments. When ^{32}P-labeled DNA was incubated with or without GST-Vp3 at various NaCl concentrations and separated from protein-free DNA by gel filtration, DNA binding was detected up to 1 M NaCl (data not shown).

Characterization of Nucleic Acid Binding—The above results suggest that the interaction of the Vp3 DNA binding domain with DNA is sequence nonspecific. We thus used a filter binding assay to examine the extent of nucleic acid binding to GST-Vp3. GST-Vp3 bound to SV40 and pBR322 DNA with identical apparent K_d values of 2.5×10^{-8} M (Fig. 4*A*). In competition experiments using unlabeled nucleic acids to compete for the binding of ^{32}P-labeled SV40 DNA (Fig. 4*B*), unlabeled SV40 DNA, pBR322 DNA, and tRNA all showed the same degree of competition, with a competitor to probe ratio of 80 to 1 (w/w) giving 50% inhibition. In contrast, single-stranded DNA, and both SV40-derived transcripts and SP6 transcripts inhibited DNA binding at ratios that were about 30-fold lower than those of unlabeled duplex DNA (Fig. 4*B*). Thus GST-Vp3 bound slightly better to RNA and single-stranded DNA than to double-stranded DNA. This was corroborated by filter binding assays using ^{32}P-labeled RNA: GST-Vp3 bound to viral and nonviral RNAs equally well with an apparent K_d of 6.6×10^{-9} M (Fig. 4*A*).

Using the same point mutants and truncated GST-Vp3 fusion proteins as for the DNA binding experiments, we probed proteins immobilized on nitrocellulose with ^{32}P-labeled RNA. Wild type Vp3, as well as all of the NTS point mutants, bound RNA, while GST-Vp3ΔC13 and GST-Vp3ΔC35 did not bind RNA (not shown). Thus the same domain that is required for DNA binding is also responsible for RNA binding. We also performed a Northwestern blot using virion-derived Vp3 and found that it too was able to bind RNA (not shown). Thus the last 35 residues of Vp3 constitutes a general nucleic acid binding domain that shows no sequence specificity for either DNA or RNA.

DNA Binding Activity of the Vp3 Carboxyl 40 Residues

Interaction of Vp3-β-Galactosidase Fusion Proteins with a 179-bp DNA Fragment—The sequence nonspecific DNA binding activity of the carboxyl-terminal 40 residues of Vp3 was next tested. Three β-galactosidase-Vp3 fusion proteins were used (19): two proteins, F6 and F6$_{202T}$, contained the carboxyl-terminal 40 residues of Vp3 and its mutant Vp3$_{202T}$

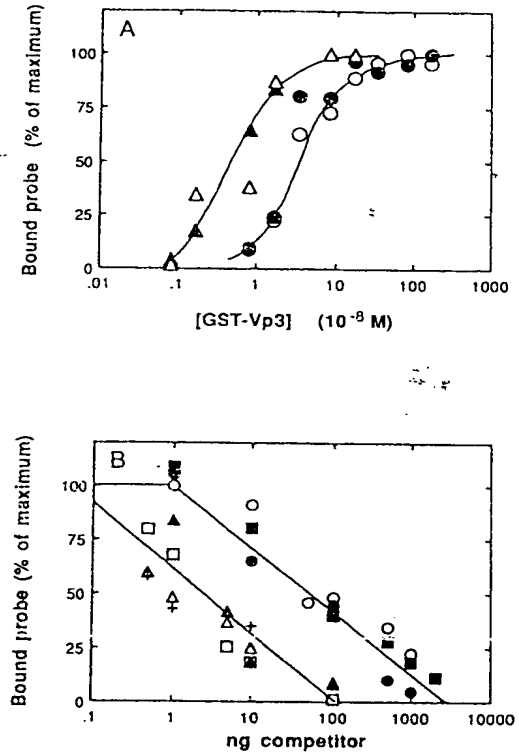

FIG. 4. **Binding of nucleic acids to GST-Vp3.** *A*, filter binding assays were performed using 1 ng of ^{32}P-labeled probe and affinity-purified GST-Vp3 as described under "Materials and Methods." A maximum of 98% of the input counts was bound and corresponds to 100% relative binding. Symbols: ○, SV40 DNA; ●, pBR322 DNA; △, Vp1 RNA; ▲, SP64 RNA. *B*, competition experiments were performed by incubating competitor nucleic acids with GST-Vp3 using 1 ng of ^{32}P-labeled SV40 DNA as probe. Binding in the absence of competitors (46% at 0.16 μM GST-Vp3) was taken as 100%. Symbols: ○, SV40 DNA; ●, pBR322 DNA; □, single-stranded M13 mp18 DNA; ■, tRNA; △, Vp3 RNA; ▲, SP64 RNA; +, Vp1 RNA. Assays in *A* and *B* were performed in duplicate and the standard deviation of all points falls within the dimensions of the symbols.

counterpart, respectively, and the third protein, E1, contained the internal 150 amino acids (45–194) of Vp3. These fusion proteins did not bind detectable DNA by the SDS-PAGE-Southwestern assay, but F6 and $F6_{202T}$ did bind DNA slightly after nondenaturing-PAGE and blotting (data not shown). These results suggested to us that the fusion proteins required nondenaturing conditions for DNA binding. We thus performed the mobility shift assay with a 179-bp ^{32}P-end-labeled DNA fragment.

When F6 was added to the reaction, protein-free DNA (Fig. 5*A*, *lane 5*, *arrowhead*) progressively disappeared as the amount of fusion protein in the reaction mixture was increased (Fig. 5*A*, *lanes 4* to *1*). Bands and smears produced by the interaction of the protein with the DNA can be seen migrating with decreased mobilities as compared to protein-free DNA. As expected, the addition of a 20-fold molar excess of unlabeled sheared *E. coli* DNA abolished the mobility shift (data not shown). When β-galactosidase or E1 was included in the reaction, little to no disappearance of the protein-free DNA was detected even though much higher concentrations of proteins were used (Fig. 5*A*, *lanes 6–10*, and *B*, *lanes 1–5*, respectively). The NTS mutant fusion protein, $F6_{202T}$, also was used in gel shift experiments and behaved similarly to F6, again demonstrating the separation of the NTS from the DNA binding domains (data not shown).

This reaction, the interaction of F6 with the double-stranded DNA fragments in solution, is a protein excess reaction, and the fraction of protein bound to DNA constitutes only a small fraction of input F6. Assuming that one F6 protein molecule binds one DNA molecule, we estimated the K_d. Half of the input DNA remained as protein-free DNA when 1.35 μg of F6 was added to the reaction mixture, giving an apparent K_d value of 2×10^{-7} M.

Binding of F6 to Long DNA Fragments—We examined the effect of fragment size on the DNA binding activity of F6. When a 1541-bp ^{32}P-labeled DNA fragment was reacted with F6, band shifts as seen in Fig. 6 were obtained. Instead of the band retardation that was seen when F6 was incubated with the shorter DNA fragment (Fig. 5*A*, *lanes 1–4*) or when GST-Vp3 was assayed by the gel shift assay using either short or long DNA fragments (Fig. 3), DNA-F6 protein complexes which migrated with increased mobilities, as compared to protein-free DNA (Fig. 6*A*, *lane 1*, *arrowhead*), could be seen (Fig. 6*A*, *lanes 2–4*, *open* and *closed circles*). When the amount of the F6 fusion protein was raised to 4 μg, a third species was seen which migrated in the gel with a reduced mobility (Fig. 6*A*, *lane 4*, *diamond*). These three discrete protein-DNA species migrated with the apparent mobilities of 0.4-, 0.85-, and 2.8-kb DNA. β-Galactosidase, purified by the same procedure as F6, did not cause a mobility shift of the 1541-bp fragment, indicating that the F6-induced mobility shift was due to the fusion protein and was not due to contaminants in the preparation (Fig. 6*B*, *lanes 8* and *9*).

We further tested if these faster migrating bands were caused by degradation of the input DNA, perhaps by a nuclease that contaminated our preparation. Reactions identical to those in *lanes 2–4* of Fig. 6 were treated further, after protein-DNA complexes had been allowed to form, with either proteinase K or SDS prior to electrophoresis. The 1541-bp fragment regained its initial mobility, as protein-free DNA, after the proteinase K treatment (Fig. 6*A*, *lanes 5–7*). Treatment of the reaction mixture with 3% SDS prior to electrophoresis had a similar effect (data not shown). We conclude that the input DNA was not being degraded, but that the faster and slower migrating DNA species were distinct complexes between F6 and DNA.

Two other sizes of labeled fragments were tested for their ability to bind F6. A 476-bp fragment behaved similarly to the 179-bp fragment resulting in bands and smears of DNA-protein complexes with decreased mobilities (Table I). When the 1014-bp DNA was used, a faster migrating DNA-protein complex was again observed that had an apparent mobility of 0.85 kb, similar, if not identical to the species seen following the interaction with the 1541-bp DNA (Table I).

Thus, the results using the β-galactosidase-Vp3 fusion proteins demonstrate that the last 40 residues of Vp3 are sufficient to bind DNA, and furthermore, that when present as a multimer, this fusion protein has the ability to perhaps compact long DNA fragments.

DISCUSSION

We have shown that Vp3, derived from SV40 virions or produced as a fusion protein in *E. coli*, is capable of binding duplex DNA as well as single-stranded nucleic acids in a sequence nonspecific manner. We have localized the nucleic acid binding domain to the carboxyl-terminal 40 amino acids of this protein and have shown that the last 13 residues are required for binding. We also have shown that this binding domain is independent from the Vp2/3 NTS. Furthermore, the last 40 residues of Vp3 are sufficient for the binding, and

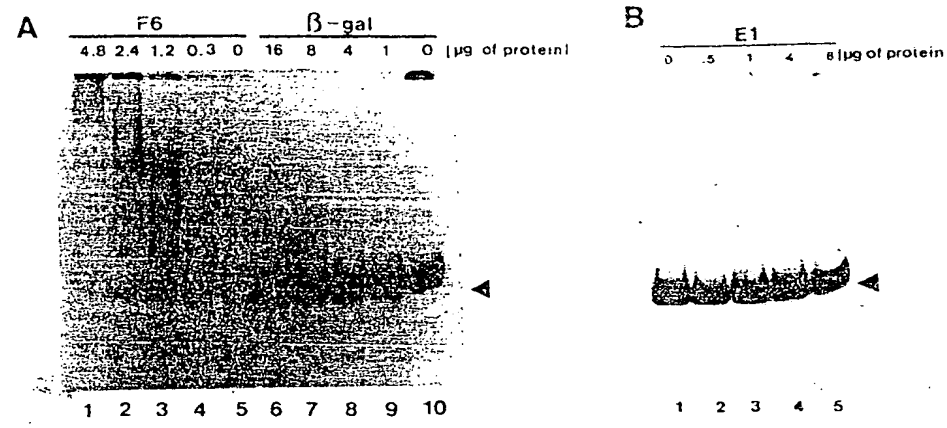

FIG. 5. **Binding of β-galactosidase-Vp3 fusion proteins with 179-bp DNA fragments.** *A*, 179-bp ^{32}P-end-labeled DNA fragments were incubated with increasing amounts of fusion protein F6 (*lanes 1–4*) or β-galactosidase (*lanes 6–9*), electrophoresed on 6% polyacrylamide gels, and subjected to autoradiography. *Lanes 5* and *10* show protein-free DNA (*arrowhead*). The reactions in *lanes 1–4* contained 4.8, 2.4, 1.2, and 0.3 μg of F6, respectively, while the reactions in *lanes 6–9* contained 16, 8, 4, and 1 μg of β-galactosidase, respectively. *B*, the ^{32}P-labeled DNA fragments were incubated with 0.5, 1, 4, and 8 μg of fusion protein E1 (*lanes 2–5*, respectively). *Lane 1*: protein-free DNA (*arrowhead*).

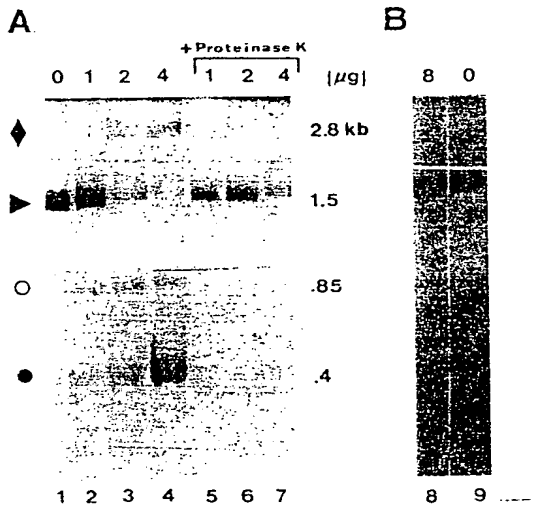

FIG. 6. **Binding of F6 to 1541-bp DNA fragments.** *A*, *lanes 1–4*, increasing amounts of F6 were incubated with a 1541-bp ^{32}P-end-labeled DNA fragment and then electrophoresed as described under "Materials and Methods." *Lane 1* shows DNA fragments incubated without F6 (*arrowhead*) while *lanes 2–4* show reactions containing 1, 2, and 4 μg of F6, respectively. The position of protein-free DNA (*arrowhead*) and discrete DNA-protein complexes, which migrated with decreased or increased mobilities compared to protein-free DNA, are marked to the *left* of the gel (*diamond*, *open circle*, and *closed circle*). The apparent mobilities of all the discrete DNA species were determined by comparison to known molecular weight markers and are shown to the right (2.8, 1.5, 0.85, and 0.4 kb). Reactions identical to those in *lanes 2–4* were further treated with proteinase K before being electrophoresed as above (*lanes 5–7*). *B*, a 1541-bp ^{32}P-end-labeled DNA fragment was incubated in the presence (*lane 8*) or the absence (*lane 9*) of 8 μg of β-galactosidase, and electrophoresed as in *A*.

TABLE I

Apparent mobilities of DNA-F6 complexes

Various sizes of end-labeled DNAs were incubated with F6 in gel mobility shift assays. Apparent mobilities were determined by comparison with molecular weight markers.

Fragment size	Apparent sizes of shifted DNA-protein complexes		
179 bp	>0.20 kb		
476 bp	>0.50 kb		
1014 bp	>1.0 kb	≈0.85 kb	
1541 bp	≈2.8 kb	≈0.85 kb	≈0.4 kb

when present as a multimeric form have the ability to compact DNA.

The substitutions of the basic residues in the Vp2/3 NTS by noncharged residues can abolish Vp2/3 nuclear localization (18, 19, 44). These mutant proteins, in the form of a mutant virion Vp2/3, GST fusion proteins, and β-galactosidase fusion proteins, bound DNA equally as well as the wild type Vp3. Thus, we effectively demonstrated that the two domains, the Vp2/3 NTS and the DNA binding domains, are separable. The results obtained using the truncated proteins localized the majority of the DNA binding domain to the carboxyl-terminal 13 residues of the protein, downstream of the NTS. However, what contributes to the residual DNA binding activity of the GST-Vp3ΔC13 is unknown. It is likely that the adjacent upstream 22 residues (from 195 to 221) are responsible for the residual activity. However, construction of the pGEX-Vp3ΔC13 plasmid resulted in the addition of 6 amino acids to the carboxyl terminus of the mutant protein, which are not normally present in Vp3, and these additional residues, LTDNSS, could contribute to the residual activity.

Our study identified a Vp2/3-DNA binding domain within the Vp3 coding sequence and 27 residues unique to Vp2 (see "Materials and Methods"), but did not test the activity in the remaining amino-terminal 91 residues of Vp2. How these residues contribute to the interaction with DNA or to overall virion assembly will be our future study. It is interesting to note that polyomavirus Vp2 and Vp3 do not contain a region similar to the carboxyl-terminal 28 residues of SV40 Vp2/3 (4). The polyoma proteins terminate immediately after the region similar to the SV40 Vp2/3-NTS (19), and it is not known whether the polyoma proteins bind to DNA. Thus the relative organization of the polyoma minichromosome and Vp2/3 molecules to Vp1 pentamers could be different from that in SV40.

The apparent K_d for DNA of the Vp3 DNA binding domain, determined by filter binding assays using GST-Vp3 was 2.5×10^{-8} M. That identical K_d values were obtained using either SV40 DNA or pBR322 DNA confirms that this interaction is sequence nonspecific. The apparent K_d for the last 40 residues of Vp3 expressed as the F6 fusion protein is about 2×10^{-7} M. This difference in affinities could be due to the multimeric nature of F6, in which the fused Vp3 residues might not be readily exposed at the surface of the fusion protein. It was found that Vp3 also binds to single-stranded DNA and RNA with roughly 4-fold greater affinity than to duplex DNA, a result similar to that reported for SV40 Vp1 (10). The nucleic acid binding K_d values of GST-Vp3 were lower than those reported for the interaction of bacterially expressed polyoma

Vp1 with DNA (10^{-11} M; 11). This is somewhat surprising since the interaction between GST-Vp3 and DNA was more stable in salt than that reported between Vp1 and DNA (11). Under alkaline conditions, SV40 Vp1 and Vp2 dissociate from the viral DNA, while most of the Vp3 and histones remain complexed with the minichromosome (13), suggesting that Vp3 might interact even more strongly than Vp1 with DNA. However, the evidence for alkaline dissociation could reflect the effect on the overall chemical bonds between the three viral proteins, and not necessarily between the viral proteins and DNA.

Several stretches within the last 35 amino acids of Vp3 share significant sequence similarity with histones H1, H2A, and H2B (17). The carboxyl-terminal 13 residues of Vp3, shown in this report to be critical for DNA binding, are 64% identical in amino acid sequence over an 11-residue stretch to a region near the amino terminus of histone H2A which is thought to make contact with the linker DNA connecting adjacent nucleosomes, thereby aiding in condensation (35, 36). Thus, this region of Vp3 might not only bind to DNA, but might also condense it in a manner similar to the H2A region in histones.

The ability of the F6 to compact DNA, as viewed by the increased mobility of long DNA fragments in the gel shift is intriguing. That the GST-Vp3 fusion protein caused only gel retardation of both short and long DNA fragments in the gel shift further suggests that the existence of the last 40 residues of Vp3 as a tetramer in F6 enables F6 to compact DNA. Judging from the result that DNA fragments shorter than 1.0 kb do not yield the fast migrating species when they interact with F6, fragment size appears to matter for compaction. We do not know how three distinct species are generated with 1.5-kb fragments, or how many F6 molecules are interacting with each fragment. This observation that F6 has the ability to change the tertiary structure of the DNA, could reflect a property of the histone-like sequences within these last 40 residues. Alternatively, it could represent a fortuitous artifact caused by a large fusion protein containing four histone-like 40-residue sequences. These species could potentially be generated by the compaction of the long fragments with F6 as well as the interaction of more than one F6-DNA complex. We attempted to visualize these three species of F6-DNA complexes by electron microscopy. After glutaraldehyde fixation of the complexes and passage through a gel filtration column, the fractions were examined by the aqueous procedure of Davis *et al.* (37). The lengths of F6-DNA complexes as well as protein-free DNA were measured by goniometry. The presence of F6 resulted in a 3-fold reduction in length compared to the protein-free DNA prepared in a similar manner (44). However, we could not resolve the structure of each of the three species of F6-DNA complexes seen in the mobility shift assays. Similar observations were made with the 5243-bp linear duplex SV40 DNA (44).

Two signals, a region required for DNA-binding and a Vp1-interactive determinant, now have been mapped to the same carboxyl 13 residues of Vp2/3 (12, 18). Whether the two are functionally and genetically inseparable or independent from each other is unknown. In either case, six DNA binding domains, five from a Vp1 pentamer and one from either Vp2 or Vp3, are expected to be involved in the compaction of an SV40 minichromosome. Whether the structural proteins bind to the minichromosome as a complex or sequentially is unknown. An *in vitro* experiment in which the conversion of polyoma Vp1 pentamers into virion-like particles, in the absence of Vp3, is blocked in the presence of DNA suggests that Vp3 may mediate the encapsidation of the minichromosome by Vp1 (11). Thus, we propose that the SV40 minichromosome interacts first with Vp2 and Vp3 molecules through a Vp2/3-DNA binding signal to nucleate the minichromosome compaction. Interaction of this complex with five Vp1-DNA binding domains within a pentamer could position the pentamer relative to the minichromosome allowing the propagation of the assembly process. Our interpretation that virion formation occurs in this distinct order is in agreement with proposed models involving progressive virion assembly (16, 38–43).

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Two Independent Signals, a Nuclear Localization Signal and a Vp1-Interactive Signal, Reside within the Carboxy-35 Amino Acids of SV40 Vp3

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The carboxy-terminal 35 amino acids (numbering 199 to 234) of SV40 Vp3 are essential for the nuclear localization of the protein as well as for its interactions with Vp1. Here, we describe studies directed at the further mapping of these two functions. Deletion and site-directed mutants of Vp3 were created within both a eukaryotic transfection and an SP6 transcription vector which encode Vp3. The subcellular localization of mutant Vp3's was assayed by immunofluorescence microscopy following DNA transfections, and the Vp1-interactive determinant of Vp3 was mapped by a recently described eukaryotic *in vitro* translation/interaction system. We show that a plasmid-encoded wild-type Vp3, whose overlapping Vp1 coding segment has been removed by mutagenesis, continues to localize to the nucleus in the absence of any SV40 Vp1. Thus, Vp3 is capable of nuclear localization on its own. Modification of Lys-202 of Vp3 into Thr is sufficient to destroy the wild-type nuclear localization of the protein, but has no effect on its interactions with Vp1. Furthermore, deletion of the terminal 13 amino acids, 222 to 234, of Vp3 does not affect its wild-type nuclear localization, but is sufficient to destroy its interactions with Vp1. Thus, the Vp3 amino acids 199–221—specifically Lys-202—are important for its nuclear localization, while the Vp3 amino acids 222–234 play a role in its interactions with Vp1. Thus, the two functions, a Vp3 nuclear localization signal and a Vp1-interactive determinant, are spatially and functionally separable within the last 35 residues of Vp3 and are, hence, independent.

INTRODUCTION

The three structural polypeptides of simian virus 40 (SV40), Vp1, Vp2, and Vp3, are synthesized in the host cytoplasm late during permissive infection. Shortly after synthesis, they are rapidly transported into the cell nucleus where viral assembly takes place (Tegtmeyer *et al.*, 1974; Lin *et al.*, 1984, 1986). In the nucleus, these structural proteins form an icosahedral capsid enclosing the viral DNA and cellular core histones (Fernandez-Munoz *et al.*, 1979; La Bella and Vesco, 1980; Tooze, 1981). As in other papovaviruses, the icosahedral symmetry of the capsid arises from pentamers of the major coat protein Vp1 (Rayment *et al.*, 1982; Baker *et al.*, 1988), which interact noncovalently with the minor coat proteins Vp2 and Vp3 (Walter and Deppert, 1974). The site at which these viral proteins interact following the synthesis of individual molecules is not known. Our previously reported genetic and biochemical data led us to propose that the viral structural proteins which are destined to form the mature virion in the nucleus commence their assembly in the cytoplasm (Kasamatsu and Nehorayan, 1979; Lin *et al.*, 1984). According to this model, Vp1 oligomers and the oligomers associated with Vp2 or Vp3 constitute precursors which translocate across the nuclear envelope. Thus, nuclear localization of Vp2 and Vp3 in infected cells could be achieved by having: (1) a Vp1 nuclear localization signal (Vp1-NLS), (2) a determinant for the Vp1-interactive domain on both Vp2 and Vp3 molecules, and (3) interaction of Vp2/3 with Vp1 multimers. In fact, both the Vp1-NLS and the Vp1-interactive domain of Vp3 have been recently mapped. The Vp1-interactive domain of Vp3 resides within the carboxyl 35 residues (Gharakhanian *et al.*, 1988). The region is essential and sufficient for the formation of Vp1:Vp3 complexes *in vitro*. The Vp1-NLS has been mapped to the first eight amino-terminal residues (APTKRKGS) of SV40 Vp1; attachment of these amino acids to the normally cytosolic capsid protein Vp1 of poliovirus promotes the nuclear localization of the fusion protein (Wychowski *et al.*, 1986).

Previous experiments from our laboratory indicated that Vp3 possesses its own NLS, just as Vp1 possesses a Vp1-NLS; Vp3 continues to localize to the nucleus in the absence of Vp2 and/or intact Vp1, as well as in the absence of agnoprotein, but is dependent on the presence of the carboxy-terminal 35 residues of Vp3 (Gharakhanian *et al.*, 1987). Note that Vp2 contains all of the amino acids of Vp3 plus 118 additional amino-terminal residues. Therefore, we presume that the same NLS functions for Vp2. Work in another labora-

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tory has indicated that the nuclear localization of an SV40 Vp2–poliovirus Vp1 fusion protein appears to be dependent on the presence of the segment 317 to 323 (KKKRK) of SV40 Vp2 (corresponding to residues 2 to 205 of SV40 Vp3) (Wychowski *et al.*, 1987), in agreement with our result; however, these authors argue that interaction with Vp1 is also important for the nuclear localization of the fusion protein. Further consideration of these issues is presented under Discussion.

In this study, we directly address the evidence for the presence of an independent Vp3-NLS, and for the spatial relationship between the Vp3-NLS and the Vp1-interactive determinant. We have introduced several mutations within the carboxy-terminal 35 residues of Vp3 to map two functions, a Vp3-NLS and a Vp1-interactive determinant. We show that: (1) the Vp3-NLS is indeed present, residing in residues 199–221 and specifically including Lys-202; (2) the Vp1-interactive domain of Vp3 lies downstream of the Vp3-NLS within Vp3 residues 222–234; (3) the two domains are functionally and spatially separable and, hence, independent; and (4) unequivocally, Vp3 localizes to the nucleus independent of Vp1.

MATERIALS AND METHODS

Construction of Vp3 mutant transfection plasmids

Various wild-type and mutant segments of SV40 DNA were placed into the pSVp23A transfection vector as described below. This plasmid has been described previously (Gharakhanian *et al.*, 1987) and encodes the 40 large T and small t antigens as well as agnoprotein, Vp2, Vp3, and the amino-terminal 96 amino acids of Vp1.

pSVp23A$_{202T}$. The Lys-202 codon of Vp3 was changed to a Thr codon (AAG → AAC) using oligo-directed mutagenesis (Fig. 1). The *Kpn*I–*Eco*RI (294–1782) fragment of SV40 was ligated into the multicloning site of mp19. Single-stranded mp19Vp123 was subjected to oligo-directed mutagenesis using the 5′CAACTTCCTTTTCGTTTTGTT antisense primer (wild-type antisense sequence: 5′CAACTTCCTTTTCCTTTTGTT) to yield mp19Vp123$_{202T}$. The *Kpn*I–*Eco*RI fragment in mp19Vp123$_{202T}$ was then exchanged with its wild-type counterpart in pSVp23A to yield pSVp23A$_{202T}$.

pSVp23AΔVp1. The initiating AUG codon of Vp1, along with another AUG triplet 6 bases upstream of the initiating one, was altered to Thr (ACG) by oligo-directed mutagenesis (Fig. 1). The second AUG was altered to ensure that the initiation from the upstream triplet does not occur. The codon change does not alter the overlapping Vp3 amino acid sequence. Single-stranded mp19Vp123 was subjected to oligo-directed mutagenesis using the 5′GGGGCCGTCTTCGTAAGCTTT antisense primer (wild-type antisense sequence: 5′GGGGCCATCTTCATAAGCTTT) to yield mp19Vp23, from which pSVp23AΔVp1 was constructed by exchanging the *Kpn*I–*Eco*RI fragment (Fig. 1).

pSVp23A$_{202T}$ΔVp1. This pSVp23A derivative contains both the Thr$_{202}$ mutation in Vp3 and the mutations described in pSVp23AΔVp1 (Fig. 1). Two primers described above were included during the mutagenesis reaction to yield mp19Vp23$_{202T}$ and pSVp23A$_{202T}$-ΔVp1.

pSVp23AΔC13. SV40 DNA was linearized with *Acc*I and was subjected to nuclease *Bal*31 digestion and linker ligation as described previously (Gharakhanian *et al.*, 1987), except that the *Bal*31 digestion was reduced to 20 min. The extent of the deletion created by the *Bal*31 digest was determined by inserting the *Eco*RI–*Eco*RV fragment of pSVp23AΔC13 into M13mp9 through its *Eco*RI and *Hin*cII sites and by sequencing (data not shown). The deletion results in a truncated Vp3 lacking its carboxy-13 amino acids.

Construction of Sp6 transcription vectors

Various wild-type and mutant segments of SV40 DNA were placed into the pSP64 transcription vector (Melton *et al.*, 1984).

Sp6Vp1 and Sp6Vp3. The plasmids have been described previously (Gharakhanian *et al.*, 1988) and encode an intact Vp1 and an intact Vp3, respectively.

Sp6Vp3ΔC13. This plasmid was constructed by exchanging the *Pfl*mI–*Eco*RI fragment of Sp6Vp3 (SV40 1007–1716) with that of pSVp23ΔC13 (SV40 1007–1575, described above). Thus, the vector encodes a truncated Vp3, Vp3ΔC13, lacking its carboxy-13 amino acids.

Sp6Vp3-202T. This vector was constructed by exchanging the *Pfl*mI–*Acc*I fragment of Sp6Vp3 (SV40 1007–1628) with that of pSVp23A-201T. Following standard purification procedures, each of the SP6Vp plasmid DNAs was treated with SDS and subjected to an additional banding in cesium chloride for further purification. Prior to *in vitro* transcriptions, Sp6Vp1 was linearized with *Sst*I, while Sp6Vp3, Sp6Vp3ΔC13, and Sp6Vp3-202T were each linearized with *Eco*RI.

DNA transfections

Culture conditions, as well as experimental details of transfection and immunofluorescence microscopy, have been described elsewhere (Gharakhanian *et al.*, 1987). Briefly, TC7 cells were subjected to DEAE-Dex-

tran mediated transfection (Lopata *et al.*, 1984), using the appropriate DNA transfection vectors. Harvested cells were subjected to immunofluorescence microscopy using polyclonal anti-Vp3 antibodies followed by fluorescein-conjugated goat anti-rabbit antibody (Cappel).

Oligo-directed mutagenesis

The site-directed mutagenesis was carried out according to the method described by Eckstein and coworkers (Taylor *et al.*, 1985) using the Amersham mutagenesis kit. In this method, selective removal of the nonmutant strand is made possible by the incorporation of a thionucleotide into the mutant strand during the extension reaction. The buffer conditions of the mutagenesis reactions are the proprietary information of Amersham. However, a brief description of reagents and reagent concentrations is as follows. Eight picomoles of phosphorylated oligonucleotides was annealed to 10 μg of single-strand template in a total volume of 34 μl of buffer at 70° for 3 min followed by 37° for 30 min. The annealed primers were extended with Klenow polymerase at 0.12 U/μl in the presence of 0.89 m*M* each of dATP, dGTP, and dTTP and 0.89 m*M* dCTPαS and ligated with T_4 DNA ligase at 0.12 U/μl in a one-step reaction in the appropriate buffer for 15 hr at 16° to generate mutant wild-type heteroduplex DNA. Following extension and ligation, unextended single-strand DNAs were removed by passing the reaction mixtures through a nitrocellulose filter (0.45-μm pore size), and the filtrates were ethanol precipitated. Following resuspension in the appropriate buffer, the heteroduplex DNAs were digested with *Ava*I at 0.5 U/μl. Thionucleotide strands are resistant to *Ava*I digestion, resulting in only single-strand nicks in the wild-type strand. Exonuclease III digestion at 1.2 U/μl for 30 min at 37° removed the nicked wild-type strand. Mutant homoduplexes were obtained by gap filling and ligation using Klenow polymerase and T_4 DNA ligase as described above except that dCTPαS is replaced with dCTP. A 0.1-vol sample of each reaction was used directly for transformation of $CaCl_2$-competent JM101 cells (0.2 ml). For the construction of pSVp23ALys-202, pSVp23AΔVpI, and pSVp23ALys-202ΔVpI the custom-made primers 5'CAACTTCCTTTTCGTTTTGTT or 5'GGGGCCGTCTTCGTAAGCTTT or both were used, respectively (underline denotes changes from the wild type), to create point mutations within an SV40 *Kpn*I/294–*Eco*RI/1782 fragment (Fig. 1). Following mutagenesis, single-strand templates were prepared from corresponding mp19 plaques and their DNA sequences were confirmed by dideoxy sequencing (Sanger *et al.*, 1980) without prior screenings. The technique yielded an efficiency of 85% for the single primer mutagenesis and 45% for the double primer mutagenesis.

Antisera

Polyclonal antisera against SDS-denatured SV40 Vp1 and/or Vp3 have been described previously (Kasamatsu and Nehorayan, 1979). Affinity-purified anti-Vp3 was the kind gift of Dr. Brenda Fung and was prepared by the procedure described by Olmsted (1981) using SDS–PAGE-purified Vp3.

In vitro transcription and capping

Transcription and capping was carried out as described previously (Gharakhanian *et al.*, 1988). Briefly, either *Sst*1- or *Eco*RI-linearized templates (1 μg) were transcribed as described by Melton *et al.* (1984) at 40° for 1 hr, except that ^{7me}GpppG (Pharmacia) was included at 500 μ*M* and the GTP concentration was lowered to 100 μ*M*.

Cell-free translation

One-third of each transcription product (1 μg) was added to 25 μl of rabbit reticulocyte lysate (Pelham and Jackson, 1976) (Promega Biotech) in the presence of 150 m*M* potassium acetate, 1 m*M* magnesium acetate, 7 units of RNasin, 50 μCi of [^{35}S]methionine (1500 Ci/mmol, NEN), and 2 μl of translation cocktail (1 m*M* DTT, 1 m*M* each of amino acids minus methionine) t a total volume of 50 μl. Cotranslations of Vp1 with wild type or mutant Vp3's were carried out in the presence of 1 μg of each RNA species. Translations were carried out for 1 hr at 30°.

Protein–protein interaction assays

Interactions among Vp3 products (wild type or mutant) or between Vp1 and Vp3 (wild type or mutan were assayed as described by Gharakhanian *et a* (1988). Occurrence of protein–protein interactions was determined by coimmunoprecipitation of two proteins (for example, coprecipitation of Vp1 with Vp3 using affinity-purified anti-Vp3 IgG), or by the sedimentation profiles of protein complexes in sucrose gradients as revealed by immunoprecipitation of the proteins with anti-Vp1 and anti-Vp3 sera followed by SDS–PAGE analyses and fluorography. In some cases, proteins gradient fractions were immunoprecipitated wi affinity-purified anti-Vp3 IgG.

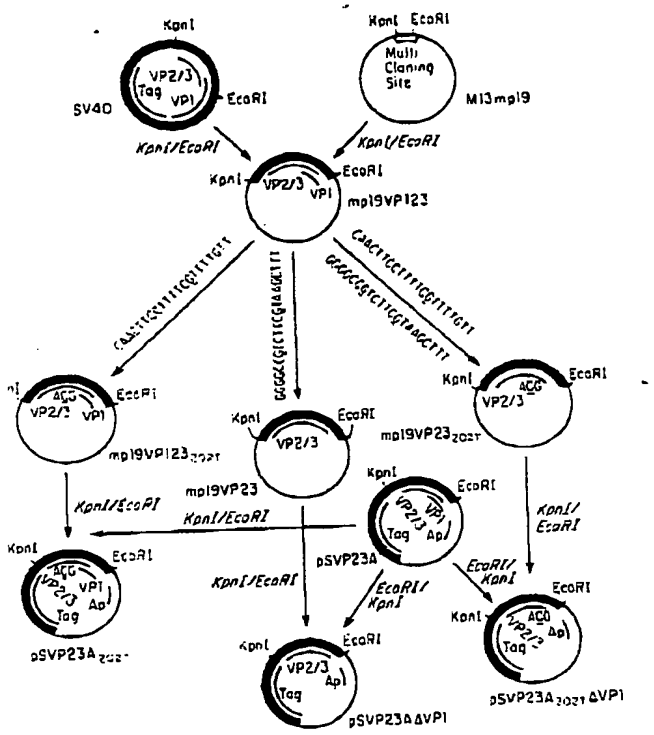

FIG. 1. Construction of SV40 Vp3 mutant transfection plasmids. Plasmids pSVp23A$_{202T}$, pSVp23AΔVp1, and pSVp23A$_{202T}$ΔVp1 were constructed as indicated (see Materials and Methods for details).

RESULTS

Identification and localization of the Vp3-NLS on Vp3

Effect of Vp1 on the nuclear localization of Vp3. Transfection experiments, using plasmids described in our previous study (Gharakhanian *et al.*, 1987), led to the encoding of up to 98 amino acids (pSVp23A) of the amino terminus of Vp1 due to the overlapping nature of the Vp3 and Vp1 coding sequences. To directly eliminate the contribution of truncated Vp1 to the nuclear-transport of Vp3, the transfection vector pSVp23AΔVp1 was constructed wherein the initiating AUG codon of Vp1, as well as an AUG appearing near the initiating codon, was destroyed (Fig. 1). The second of two AUG codons appearing within 9 base pairs of each other has been identified as the Vp1 initiating codon (Tooze, 1981). On the basis of Kozak's rule that the surrounding sequence context of an AUG triplet influences the initiation of eukaryotic protein synthesis (Kozak, 1986), we conclude that the first AUG, appearing two triplets upstream of the Vp1 initiating codon, has an unfavorable context for initiation; however, it could be recognized by ribosomes in the absence of the Vp1 initiating codon. Thus, both AUG codons were mutated to ensure no initiation of the truncated Vp1. The oligo-directed mutagenesis was designed such that the overlapping Vp3 bases continued to encode wild-type Vp3 amino acids. The transfection plasmid (pSVp23AΔVp1) encodes only Vp2 and Vp3 as well as agnoprotein. The pSVp23AΔVp1 DNA was introduced into TC7 cells by DNA transfection and the subcellular localization of Vp2/3 was determined by indirect immunofluorescence microscopy using polyclonal anti-Vp3 antiserum.

As shown in Fig. 2C, the nuclear localization of Vp2 and Vp3 in the pSVp23AΔVp1 transfected cells was identical to that in the pSVp23A transfected cells (Fig. 2A). Together with our previously reported results that agnoprotein does not play a role in the nuclear localization of Vp2 and Vp3 and that elimination of the Vp2 initiating AUG codon does not affect the nuclear localization of Vp3 (Gharakhanian *et al.*, 1987), this result indicates that Vp3 is capable of nuclear localization on its own.

Vp3-NLS within residues 199 to 221. The carboxyl 35 residues of Vp3, previously shown to be important for its nuclear localization (Gharakhanian *et al.*, 1987), include a stretch of basic residues (PNKKKRKL) similar in sequence to the nuclear transport signal of SV40 T antigen (Kalderon *et al.*, 1984a,b; Lanford and Butel, 1984). Furthermore, the same carboxyl 35 residues of Vp3 have been shown to contain a Vp1-interactive domain as determined by an *in vitro* protein–protein interaction assay (Gharakhanian *et al.*, 1988). In order to further map the Vp3-NLS within the carboxyl 35 residues, three transfection plasmids, pSVp23AΔC13, pSVp23A$_{202T}$, and pSVp23A$_{202T}$ΔVp1, were constructed. pSVp23AΔC13 was constructed via *Bal*31 nuclease digestion and encodes a truncated Vp3 missing its carboxyl 13 amino acid residues (see Materials and Methods). The plasmids pSVp23A$_{202T}$ and pSVp23A$_{202T}$ΔVp1 were constructed via site-directed mutagenesis using synthetic oligonucleotides (Fig. 1). In pSVp23A$_{202T}$, the lysine 202 residue was mutated to threonine. This Lys-202 of Vp3 is situated in a position equivalent to the Lys-128 of the SV40 T antigen NLS whose mutation to Thr-128 is sufficient to alter the subcellular localization of the mutant T antigen from the nucleus to the cytoplasm (Lanford and Butel, 1984; Kalderon *et al.*, 1984a,b). Furthermore, modification of the same residue in SV40 Vp2 affects the nuclear localization of SV40 Vp2/3–poliovirus Vp1 fusion proteins (Wychowski *et al.*, 1987). In pSVp23A$_{202T}$ΔVp1, three independent base substitutions were introduced. This

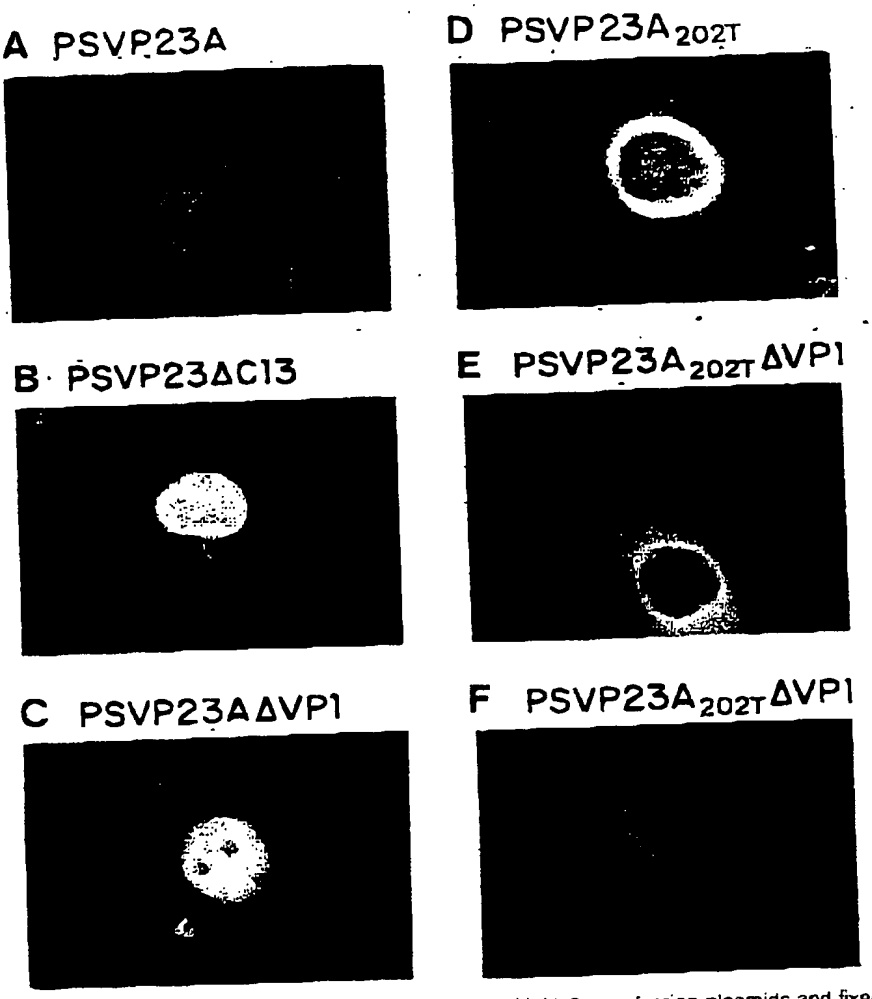

FIG. 2. Subcellular localization of mutant Vp3. TC7 cells were transfected with Vp3 transfection plasmids and fixed 48 hr after transfection. Cells were reacted with anti-Vp3 sera followed by FITC-conjugated secondary antibody as described under Materials and Methods. The SV40 plasmid used for transfection is indicated above each photograph. The nuclear staining obtained with pSVp23A (A), pSVp23A$_{\Delta C13}$ (B), and pSVp23AΔVp1 (C) are similar to those obtained with wild-type SV40 transfections. The cytoplasmic and perinuclear staining obtained with pSVp23A$_{202T}$ (D) and pSVp23A$_{202T}$ΔVp1 (E and F), however, is not seen in wild-type transfections.

plasmid does not encode the truncated Vp1, which is present in pSVp23A$_{202T}$, but does encode a mutant Vp3 where the Lys-202 is altered to a Thr (Fig. 1).

TC7 cells were transfected with the mutant plasmid DNAs, and the subcellular localization of mutant Vp3's was determined immunocytochemically using anti-Vp3 sera. Without exception, the truncated Vp3$_{\Delta C13}$ was localized to the nucleus (Fig. 2B). Thus, Vp3 is capable of nuclear localization in the absence of its carboxyl 13 residues. In contrast to the nuclear localization of Vp3$_{\Delta C13}$, a mutation at the 202 residue of Vp3 from Lys to Thr altered the subcellular localization of the mutant Vp3 expressed in pSVp23A$_{202T}$ transfected cells. In all the cells exhibiting a positive Vp3 staining, mutant Vp3 was limited to a cytoplasmic and perinuclear distribution (Fig. 2D). The identical Vp3 staining pattern was observed in pSVp23A$_{202T}$ΔVp1 transfected cells (Figs. 2E and 2F), confirming the importance of the Lys-202 residue of Vp3 for its nuclear localization. These results not only indicate the presence of an independent Vp3-NLS but also map the Vp3-NLS to within a stretch of 23 amino acids spanning residues 199 to 221 of Vp3.

Localization of the Vp1-interactive domain on Vp3

The carboxyl 40 amino acids of Vp3 (aa 195–234) are essential and sufficient for its interactions with Vp1 (Gharakhanian *et al.*, 1988). We have now established that a single mutation at Lys-202 of Vp3 to Thr is suffi-

cient to destroy the nuclear localization of the protein, but that the terminal 13 residues have no role in its nuclear localization. Thus, at least two functions, coding for the Vp3-NLS and the Vp1-interaction domain, reside in the carboxyl end of Vp3. To test whether the two functions are physically separable within that region, we have examined the interaction of Vp1 with nuclear localization defective Vp3 ($Vp3_{202T}$), or with truncated Vp3 missing its carboxyl 13 residues ($Vp3_{\Delta C13}$).

The Vp3 coding sequence in either pSVp23A_{202T} or pSVp23AΔC13 was exchanged with that of the transcription plasmid SP6Vp3, rendering SP6$Vp3_{202T}$ and SP6Vp3ΔC13, respectively (see Materials and Methods). Synthetic mRNAs coding for mutant $Vp3_{202T}$ or $Vp3_{\Delta C13}$ were prepared by the transcription of the plasmid DNAs with SP6 RNA polymerase and were translated alone or along with synthetic Vp1 mRNA derived from SP6 Vp1, using rabbit reticulocyte lysates. The translation products were allowed to interact for an additional 2 hr at 30° and were then sedimented through sucrose gradients. Whether mutant Vp3 interacts with Vp1 was examined by immunoprecipitation of each fraction with anti-Vp3 sera followed by SDS–PAGE analyses.

As shown in Fig. 3A, the mutant $Vp3_{202T}$ sedimented as a 4–6 S species (by comparison with the BSA, 4.5 S, and IgG, 7 S, markers). When mutant Vp3 was cotranslated with Vp1 and the products incubated for 2 hr, the $Vp3_{202T}$ sedimented as a heterogenous species with values of 9 through 12 S, which is in a region of the gradient where Vp1 oligomers were also found (Fig. 3B). Thus, the sedimentation profile shift for $Vp3_{202T}$ following incubation with Vp1 is the same as that observed following the incubation of wild-type Vp3 with Vp1 (Gharakhanian *et al.*, 1988).

If the Vp1 and $Vp3_{202T}$ bands seen in fractions 5 to 11 of Fig. 3B represent a cosedimentation of the two proteins due to protein–protein interaction, it should be possible to immunoprecipitate the protein complex using antibodies against only one component of the complex. When proteins in fraction 9 of Fig. 3B (arrow) were immunoreacted with affinity-purified anti-Vp3 IgG, Vp1 coprecipitated with the mutant $Vp3_{202T}$ (Fig. 4, lane 1). This result further confirms the interaction between mutant $Vp3_{202T}$ and wild-type Vp1. Thus, the mutant $Vp3_{202T}$ protein is capable of interaction with Vp1, although it is deficient in nuclear localization.

The majority of $Vp3_{\Delta C13}$, analogous to $Vp3_{202T}$ (and wild-type Vp3), sedimented as a 4 to 6 S species after post-translational incubation (Fig. 3C), but a minor fraction sedimented at 7 to 8 S. In contrast to the S value shift observed due to the interaction of Vp1 with

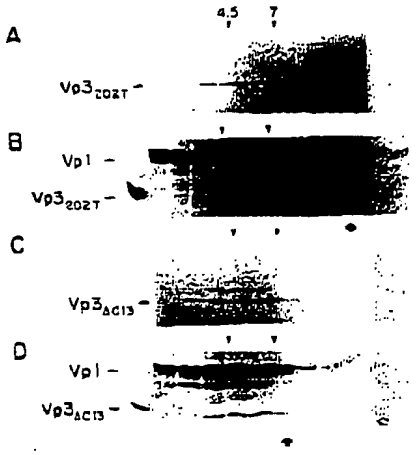

FIG. 3. Interaction of $Vp3_{202T}$ or $Vp3_{\Delta C13}$ with Vp1. (A and C) [^{35}S]-methionine labeled, *in vitro* translated $Vp3_{202T}$ (A) or $Vp3_{\Delta C13}$ (C) were subjected to sucrose gradient sedimentation following a 2-hr post-translational incubation (see Materials and Methods). Fractions were immunoprecipitated with anti-Vp3 sera and precipitates subjected to SDS–PAGE followed by fluorography. (B and D) [^{35}S]methionine labeled, cotranslation products of Vp1 and $Vp3_{202T}$ (B) or Vp1 and $Vp3_{\Delta C13}$ (D) were allowed to interact for 2 hr following translation and were subjected to sucrose gradient sedimentation. Proteins in fractions were immunoprecipitated with anti-Vp3 and anti-Vp1 sera, and immunoprecipitates were analyzed by SDS–PAGE and fluorography. The first lane at the left contains ^{35}S-labeled $Vp3_{202T}$ (B) or $Vp3_{\Delta C13}$ (D) as a marker. Sedimentation markers (BSA, 4.5 S; IgG, 7 S) are indicated at the top of each panel. The top 11 fractions of 14 are shown in the figure; the bottom 3 fractions of each were devoid of labeled proteins. The arrows in (B) and (D) indicate the fraction used for analysis of coprecipitation with affinity-purified anti-Vp3 IgG (Fig. 5).

$Vp3_{202T}$ (and wild-type Vp3), an S value shift was not observed when $Vp3_{\Delta C13}$ was incubated with Vp1 (Fig. 3D): $Vp3_{\Delta C13}$ sedimented as a 4 to 8 S species (fractions 2 through 8) whether the mutant Vp3 was incubated alone (Fig. 3C) or with Vp1 (Fig. 3D). Although we noted the presence of both Vp1 and $Vp3_{\Delta C13}$ proteins in fractions 6–8 of the sucrose gradient (Fig. 3D), the two proteins were not in association with one another. There was no detectable Vp1 coprecipitating with the mutant Vp3 when a fraction containing both $Vp3_{\Delta C13}$ and Vp1 species (sixth fraction from the top, arrow in Fig. 3D) was immunoreacted with affinity-purified anti-Vp3 IgG (Fig. 4, lane 2). Thus, in the absence of its carboxyl 13 amino acids, a truncated Vp3 no longer interacts with Vp1. The same truncated Vp3, however, continues to localize in the nucleus (Fig. 2B). These studies show that the carboxyl terminal amino acid residues 222–234 of Vp3 play a role in its interaction with Vp1.

DISCUSSION

The 40 carboxy-terminal residues of Vp3 are necessary and sufficient for its interactions with Vp1 *in vitro*

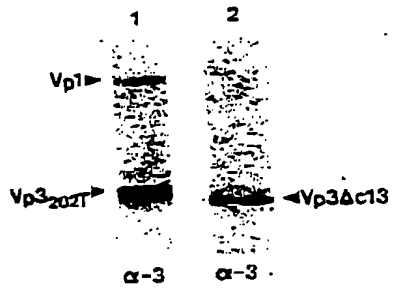

FIG. 4. Interaction of mutant Vp3 with Vp1. Radiolabeled mutant Vp3 and Vp1 were allowed to interact and were sedimented through 5/20% sucrose gradients (see Materials and Methods). Proteins in a fraction were immunoreacted with affinity-purified anti-Vp3 IgG (α-3), and the immunoprecipitates were analyzed by SDS–PAGE followed by fluorography. Lane 1: $Vp3_{202T}$ and Vp1 coincubation. The ninth fraction from the top of the gradient in Fig. 3B was immunoreacted with the IgG as described under Materials and Methods. Lane 2: $Vp3_{\Delta C13}$ and Vp1 coincubation. The sixth fraction from the top of the gradient in Fig. 3D was immunoreacted with the IgG. Positions for Vp1 and mutant Vp3 are marked at the left and right of the figure. Stoichiometry of both Vp1 and $Vp3_{202T}$ in lane 1 and in fraction 9 of Fig. 3B was determined by densitometry of autoradiograms and by correcting the number of methionine residues. Values for $Vp3_{202T}$: Vp1 are 5:1 in lane 1 and 3:1 in fraction 9, respectively. In a separate experiment, stoichiometry of wild-type Vp3 and Vp1 related proteins was obtained by densitometry analysis of protein bands obtained by reaction with the affinity-purified antibody immediately following post-translational incubation (not shown). The value for Vp3:Vp1 is 1:1.

(Gharakhanian *et al.*, 1988). It has been suggested that a domain within the same region is important for the nuclear localization of Vp3 *in vivo* (Gharakhanian *et al.*, 1987; Wychowski *et al.*, 1987). All results presented in this paper, as well as the results of our previous studies (Gharakhanian *et al.*, 1987, 1988), are summarized in Fig. 5. Our studies unambiguously show the presence of an independent Vp3-NLS and further dissect the two functions into two spatially independent units (Fig. 5). The Vp3-NLS lies within residues 199–221 and is dependent on the presence of Lys-202. The Vp1-interactive determinant of Vp3 lies downstream of its nuclear localization signal within the terminal 13 amino acids (residues 222–234). The two functions are independent of each other and can be dissected with the use of appropriate mutants (Fig. 5). A nuclear-transport-defective Vp3 continues to interact with Vp1 *in vitro* while a truncated Vp3 defective in interactions with Vp1 continues to localize to the nucleus (Fig. 5). Since both the nuclear directing and the Vp1-interactive domains are also present in SV40 Vp2, we believe they may perform the same nuclear localization and Vp1-interactive functions for Vp2.

There has been a dispute over the presence of an independent Vp3-NLS. While our results show the importance of its own carboxyl 35 residues for the nuclear localization of Vp3 (Gharakhanian *et al.*, 1987), Wychowski *et al.* (1987) have shown that the Vp3-NLS alone is not sufficient for the nuclear localization of Vp3. The loss of nuclear accumulation of truncated Vp3 in the former study (Gharakhanian *et al.*, 1987) could be accounted for by the simultaneous loss of the overlapping Vp1. Thus, an argument against the presence of an independent Vp3-NLS was raised in the latter study (Wychowski *et al.*, 1987). In their study, SV40 Vp2/3–poliovirus Vp1 fusion proteins containing the wild-type SV40 sequence PNKKKRK (Vp2 sequence 317 to 323, and Vp3 sequence 199 to 205) localize to the nucleus in the presence of an SV40 helper virus at high multiplicity of infection (m.o.i., 2–3 PFU/cell). However, at a low m.o.i. (10^{-4} PFU/cell) in which a simultaneous infection by both a recombinant virus and a helper virus is considered to be negligible, the same fusion proteins are found in the cytoplasm, and not in the nucleus. The fusion proteins do not contain the carboxyl 18 or 23 residues of SV40 Vp2/3, within which, according to our present results, the Vp1-interactive determinant of Vp3 resides. We therefore expect these fusion proteins to be unable to interact with SV40 Vp1 regardless of the multiplicity of infection. The fusion proteins do, however, contain an intact Vp3-NLS according to our interpretation. Therefore, the SV40 Vp3-NLS in the SV40 Vp2/3–poliovirus Vp1 fusion proteins should have promoted the nuclear localization of normally cytoplasmic poliovirus Vp1 and localized to the nucleus regardless of the multiplicity of infection. The reported difference in the nuclear localization of the same fusion proteins over different m.o.i. may not be simply explained by the presence of the Vp3-NLS nor by the presence or absence of an intact SV40 Vp1 expressed by the helper SV40 virus. The poliovirus Vp1's are cytoplasmic proteins (Wychowski *et al.*, 1985) and could harbor a cytoplasmic localization signal or a protein-interactive signal which promotes their retention within a cytoplasmic compartment. Attachment of an SV40 Vp3-NLS to a poliovirus Vp1 might not have been sufficient for targeting the fusion protein to the nucleus as has been seen between SV40 T antigen NLS and polyoma middle T antigen (Richardson *et al.*, 1986). Although one SV40 Vp3-NLS in individual fusion proteins may not counteract the cytoplasmic localization of poliovirus Vp1, more than one SV40 Vp3-NLS formed by the interaction of the fusion protein with one or more SV40 Vp2 or Vp3 molecules expressed by the helper virus may target the fusion protein to the nucleus. Thus, the observed SV40 Vp3-NLS phenotype could be due to the cotransport of the fusion proteins with SV40 Vp2 and Vp3; a multiprotein complex between

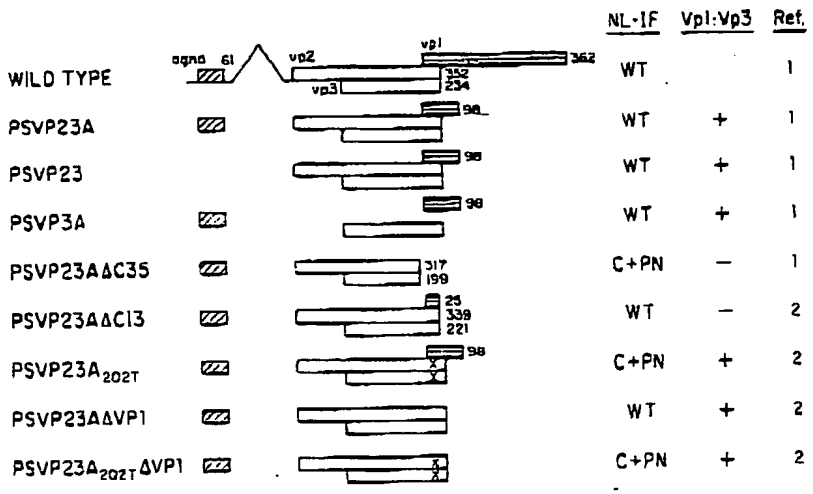

FIG. 5. Summary of mutant studies for identification of the Vp3-NLS and the Vp1-interactive domain. SV40 structural proteins expected to be produced in the cells transfected by various plasmids are shown together with the results of the nuclear localization phenotype of Vp3 or mutant Vp3 (NL-IF) in those cells as assayed by indirect immunofluorescence microscopy and of Vp1:Vp3 (or mutant Vp3) interaction assay. Plasmids constructed in this study (Ref. 2 in the figure), as well as in the reported study (Ref. 1: Gharakhanian *et al.*, 1987, 1988), are shown. The plasmids also encode SV40 large T and small t antigens: (▨) agnoprotein; (□) Vp2 and Vp3; (▤) Vp1. Mutation is marked as X. The immunofluorescent staining pattern (NL-IF) shows either wild-type staining (wt, representing a typical strong nuclear staining accompanied by weak but uniform cytoplasmic staining) or cytoplasmic and perinuclear staining (C + PN). The ability of Vp3 or mutant Vp3 to interact with wild-type Vp1, extrapolated from the results obtained in this and the previous studies (Gharakhanian *et al.*, 1988), is presented (Vp1:Vp3 column) and is expressed as + (positive interaction) or as − (lack of interaction). The column refers only to the wild-type or mutant Vp3 of a given plasmid as transcribed and translated from an Sp6-transcription vector and to the interactions of the specified Vp3 with wild-type Vp1.

poliovirus Vp1 and SV40 Vp1–SV40 Vp2/3 has been noted at high m.o.i. in their study (Wychowski *et al.*, 1987). When two regions within the Vp2 (residues 317 to 323 and 340 to 352) or Vp3 (residues 199 to 205 and 222 to 234) are simultaneously deleted in SV40-32-am404, the subcellular localization of SV40 Vp2/3-poliovirus Vp1 fusion proteins is altered to cytoplasmic even at high m.o.i. (Wychowski *et al.*, 1987). Although their interpretation differs from ours as to the presence of an independent Vp3-NLS, the data presented in this paper are in agreement with their observations. The deletion mutant has lost both the SV40 Vp3-NLS and the Vp1-interactive determinant, and thus the mutant fusion protein is unable to localize to the nucleus on its own or piggybacked by SV40 Vp1. Our result, presented here, that Vp3 can localize to the nucleus on its own, shows how Vp3 protein functions with regard to its nuclear localization when other structural proteins are absent. Previous observations presented by us (Kasamatsu and Nehorayan, 1979) and by Wychowski *et al.* (1987) on nuclear localization of Vp3 in the presence of Vp1 identify other Vp3 epitopes that are important for the Vp1 assisted nuclear localization. Whether association with Vp1 is sufficient for the transport of SV40 Vp2/3 into the nucleus can be clarified by the introduction of a nuclear-transport-defective Vp3 and wild-type Vp1 or a both nuclear-transport-defective and Vp1-interactive-defective Vp3 and wild-type Vp1 into the same cell.

The Vp3-NLS, residues 199 to 221, shows a striking homology with the nuclear transport signal of the SV40 T antigen (Kalderon *et al.*, 1984a,b; Lanford and Butel, 1984), and these NLSs are expected to utilize similar nuclear entry mechanisms through nuclear pores. The mutation at the 202 residue of Vp3 from Lys to Thr altered its subcellular localization; the mutant Vp3 localized to cytoplasmic and perinuclear regions. This Lys-202 in the Vp3-NLS is equivalent to Lys-128 of SV40 T antigen for which a Lys to Thr or Lys to Asn mutation abolished its nuclear localization and caused a uniform distribution in the cytoplasm (Kalderon *et al.*, 1984a; Lanford and Butel, 1984). This difference in the staining patterns of the two mutant proteins may be explained by the presence of additional intracellular targeting signal(s) other than the NLS in them. In the case of the Vp3 mutation, the intracellular trafficking signal is still intact and has allowed the accumulation of the mutant protein at the perinuclear region. The possibility that the mutant Vp3 localized to inside the nuclear envelope due to the lack of an intranuclear trafficking signal is ruled out; the *Escherichia coli* β-galactosidase–Vp3 fusion protein in which carboxyl 40 residues of Vp3 were

attached to the β-galactosidase localized mostly to the nucleus upon microinjection into the cytoplasm, whereas a mutant fusion protein in which the Lys-202 was mutated to Thr localized mostly to the cytoplasm (J. Clever, unpublished observation). Thus, the Vp3 mutation abolishes the translocation of the protein across the nuclear envelope.

The Vp1-interactive determinant delineated in this study is the 13 carboxy-terminal residues of Vp3 222 to 234, KARHKRRNRSSRS, and is composed of predominantly charged, basic residues. The interaction is likely to involve salt bridges between the positively charged residues in the Vp1-interactive domain of Vp3 and negatively charged residues in Vp1. At present we do not know the location or nature of a reciprocal Vp1 domain that is involved in the Vp1:Vp3 interaction.

Affinity-purified anti-Vp3 IgG coprecipitated Vp1 with $Vp3_{202T}$, the nuclear-transport-defective Vp3, following sedimentation through a sucrose gradient. This result is in good agreement with that observed for the interaction of wild-type Vp3 and Vp1 (Gharakhanian *et al.*, 1988) and indicates that the Vp3-NLS mutant maintains an intact Vp1-interactive domain. Similarly, the lack of interaction between the truncated $Vp3_{\Delta C13}$ and Vp1 is analogous to that observed between $Vp3_{\Delta C35}$ and Vp1 (Gharakhanian *et al.*, 1988). Whether the carboxyl 13 residues are sufficient for the interaction with Vp1 is not yet known (Fig. 5). We have noticed repeatedly that in our fractionation procedure the molar ratio of Vp1 to Vp3 or to $Vp3_{202T}$ is low. This is particularly noticeable in Fig. 4 (lane 1). Without sucrose gradient fractionation, the proportion of Vp1 molecules (full length as well as truncated) coprecipitated with Vp3 by the affinity-purified antibody was higher than when samples were fractionated; about one Vp1-related molecule is found per one Vp3 molecule (see legend to Fig. 4) compared to one Vp1 molecule per three $Vp3_{202T}$ molecules in fraction 9. The fact that the stoichiometry of Vp1 and Vp3 in the complex is higher in the unfractionated sample than that in the fractionated sample may suggest that the binding constant involved in the interaction is not high. Alternatively, the observed change in ratio could result from the presence of more than one kind of complex being included in the unfractionated sample but absent in fraction 9.

A noteworthy observation is the clustering of the two functionally distinct and physically separable domains within the carboxyl 40 residues of Vp3. Our results also indicate that this segment contains a DNA-binding domain: addition of the 40 residues to *E. coli* β-galactosidase promotes the DNA binding of β-galactosidase (J. Clever, unpublished observation). Thus, this short stretch of Vp3 may constitute a unique segment with functional domains that are involved in protein transport, in interaction with major coat protein Vp1, and in assembly through interaction with SV40 minichromosomes. The Vp1-NLS is located within the same-coding segment but in a different amino acid reading frame (Wychowski *et al.*, 1986). At present, how each of the two NLS signals recognizes host cellular machinery is not known, nor is the relative location of the two signals in a virion particle. An attractive view would be that the two NLS signals form a structural unit within which two signals are in proximity; this unit enhances the nuclear localization of the multimeric protein complex and is thus responsible for regulating the proper stoichiometry of individual coat proteins within the nucleus (Lin *et al.*, 1984). Experimental evidence indicates a relationship between the rate of nuclear localization and the number of NLSs: the rate of nuclear localization of both nucleoplasmin and nonnuclear proteins harboring varying numbers of the NLS is dependent on the number of NLSs (Dingwall *et al.*, 1982; Lanford *et al.*, 1986; Dworetzky *et al.*, 1988). Our preliminary results indicate that S–S-mediated covalent interaction among Vp1 molecules as well as noncovalent interactions between the structural proteins Vp1, Vp2, and Vp3 have been detected in the cytoplasm of SV40-infected cells (H. Kasamatsu, unpublished observation). If transport of the protein multimer is mediated by a pore receptor(s), a study of the physical locations of the two NLSs in the complex might shed light on understanding the dynamics of protein transport and localization into the nucleus.

ACKNOWLEDGMENTS

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Analysis of a Nuclear Localization Signal of Simian Virus 40 Major Capsid Protein Vp1

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The nuclear localization signal of the major structural protein, Vp1, of simian virus 40 was further defined by mutagenesis. The targeting activity was examined in cells microinjected with SV-Vp1 variant viral DNAs bearing either an initiation codon mutation of the agnoprotein or mutations in the Vp1 coding sequence or microinjected with pSG5-Vp1 and pSG5-Vp1 mutant DNAs in which Vp1 or mutant Vp1 is expressed from simian virus 40 early promoter. The Vp1 nuclear localization signal functioned autonomously without agnoprotein once the Vp1 protein was synthesized in the cytoplasm. The targeting activity was localized to the amino-terminal 19 residues. While replacement of cysteine 10 with glycine, alanine, or serine did not affect the activity, replacement of arginine 6 with glycine caused the cytoplasmic phenotype. When multiple mutations were introduced among residue 5, 6, 7, 16, 17, or 19, the targeting activity was found to reside in two clusters of basic residues, a cluster of lysine 5, arginine 6, and lysine 7 and a cluster of lysine 16, lysine 17, and lysine 19. The clusters are independently important for nuclear localization activity.

Once papovaviruses, a class of DNA tumor viruses, enter the cell nucleus, they spend most of their reproduction cycle there. During the late phase of infection, cytoplasmically synthesized structural proteins are transported to the nucleus, where they assemble into mature virions (24). While the nuclear localization signal (NLS) of each viral structural protein is usually responsible for the protein's nuclear localization (4, 5, 7, 10, 17, 26, 27), a localization-defective mutant Vp2/3 protein, $Vp2/3_{202T}$, of simian virus 40 (SV40) can localize to the nucleus if wild-type Vp1 is coexpressed in the same cell (13). This fact implies that some SV40 subvirion assembly must take place in the cytoplasm prior to the nuclear targeting of the structural protein. The SV40 Vp1 NLS has been mapped within the first 8 amino acid residues, which can promote nuclear localization of the poliovirus structural protein Vp1, a cytoplasmic protein (26). However, among the relatively well defined NLSs, neither 8 nor 11 amino acids of the amino terminus of SV40 Vp1 are able to promote nuclear entry of the reporter protein when the signal is chemically coupled to it (6). Chelsky et al. have argued that the SV40 Vp1 NLS may require the downstream cysteine residue 10 to maintain a specific conformation for its activity (6). As the Vp1 NLS is likely to be important not only for Vp1 nuclear targeting but also for virion nuclear targeting, we extended analysis of the Vp1 NLS by mutagenesis with an SV40 variant DNA, SV-Vp1, in which the coding segments for Vp2/3 are deleted and which expresses Vp1 (13). Our studies revealed that the Vp1 NLS is a bipartite signal composed of two tracts of basic residues between residues 5 and 19.

In addition to the capsid proteins, the late region of the SV40 genome encodes a small protein, agnoprotein, which is a 61-amino-acid, basic protein that can bind DNA (14). The agnoprotein is expressed late in infection and is present predominantly in the cytoplasmic fraction (14, 19). It has been suggested to play a role in the assembly of virions in the SV40 lytic cycle (16, 18) and in Vp1 nuclear localization (3, 20). We first examined the effect of agnoprotein on Vp1 nuclear localization. pSV-Vp1ΔAgno was constructed, as outlined in Fig. 1, by exchanging the 1,452-bp *Kpn*I-*Sac*I fragment with a PCR-generated fragment in which the initiating ATG of the agnogene was changed to TAG (Fig. 1). For the PCR, the sense primer 5′-CCGCCTCAGAAGGTACCTAACCAAGTTCCT CTTTCAGAGGTTATTTCAGGCCtaGGTGCT-3′ (nucleotides 283 to 342) and the antisense primer 5′-CAAGAATTC GAGCTCGCCCCAACTTG-3′ (designated the *Sac*I antisense primer) were used along with the pSV-Vp1 template (lowercase letters in the PCR primer sequence represent the mutations). The subcellular distributions of large T antigen and agnoprotein (Fig. 2) and of Vp1 (Fig. 3) were examined following nuclear microinjection of SV-Vp1ΔAgno and were compared with those of SV-Vp1. Large T antigen localized to the nuclei in the SV-Vp1ΔAgno-injected cells in fashion similar to that in the SV-Vp1-injected cells (Fig. 2). The perinuclear and cytoplasmic localizations of agnoprotein in SV-Vp1-injected cells (Fig. 2) were the same as those reported for SV40-infected cells (14, 19). In contrast, no agnoprotein was observed in any compartment of the mutant-injected cells (Fig. 2). Vp1 expressed from SV-Vp1ΔAgno localized to the nucleus (Fig. 3), as it did when the agnogene-containing construct, SV-Vp1, was used (Fig. 3). Thus, the observation that Vp1 can localize autonomously is in contrast with the reported role of agnoprotein in Vp1 nuclear localization in the BSC-1 cell line (3, 20). The difference could be explained by differences in the cell lines, as proposed by others (3). As the protein coding sequences in the leaders of some late mRNAs may also function in late transcriptional regulation (1, 2, 11, 12, 23), subsequent analyses of the Vp1 NLS were performed with SV-Vp1.

Progressive amino-terminal-deletion mutants of Vp1, SV-Vp1 dl (3–7) (a deletion of residues 3 through 7), SV-Vp1 dl (3–12) (a deletion of residues 3 through 12), and SV-Vp1 dl

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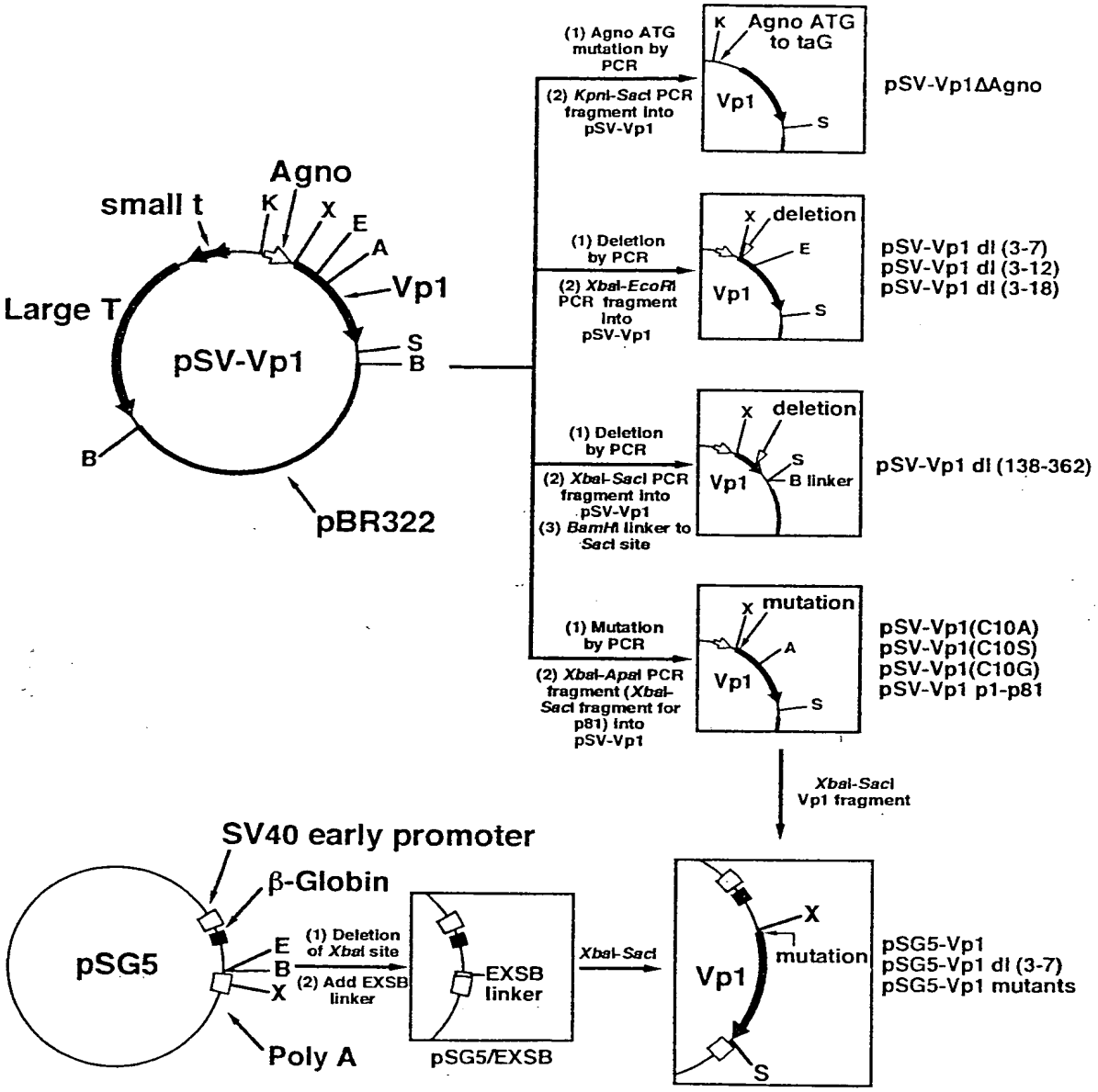

FIG. 1. Schematic diagrams for the construction of pSV-Vp1, pSG5-Vp1, and their mutant plasmid DNAs used in this study. All pSV-Vp1 constructs encode SV40 large T antigen, small t antigen, agnoprotein, and all the regulatory elements. Only the pertinent portion of each mutant plasmid is shown. All the mutations were verified by double-stranded-DNA sequencing with Sequenase version 2.0 (United States Biochemical). Oligonucleotides for PCR (sequences given in the text) and for sequencing were synthesized by the Oligonucleotide Preparation Laboratory of the UCLA Molecular Biology Institute. Following PCR-directed mutagenesis, the fragments were digested by two restriction enzymes and exchanged with those corresponding to the parent plasmids as described in the text. Abbreviations: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; S, *Sac*I; X, *Xba*I. For the construction of the pSV-Vp1 dl (138–362) mutant, a *Bam*HI linker,

5′- CGGATCCATCGATGGATCCGAGCT-3′
3′-TCGAGCCTAGGTAGCTACCTAGGC -5′

was inserted at the *Sac*I site for excision of the pBR322 sequence and for circularization. Although SV40 nucleotide sequences 2533 to 2593 (part of the coding segment of Vp1) are present, they will not be translated, since three termination codons precede these sequences. The strategy for the construction of the pSG5-Vp1-NLS mutants is as follows. The unique *Xba*I site of the mammalian expression vector pSG5 (Stratagene) was destroyed by digestion with *Xba*I, followed by a fill-in reaction of the restriction site and religation. Into this construct, an EXSB linker,

5′-AATTCTCTAGAATCGTACGATAGAGCTCG -3′
3′- GAGATCTTAGCATGCTATCTCGAGCCTAG-5′

which includes *Xba*I and *Sac*I sites, was inserted at its *Eco*RI and *Bam*HI sites to yield pSG5/EXSB.

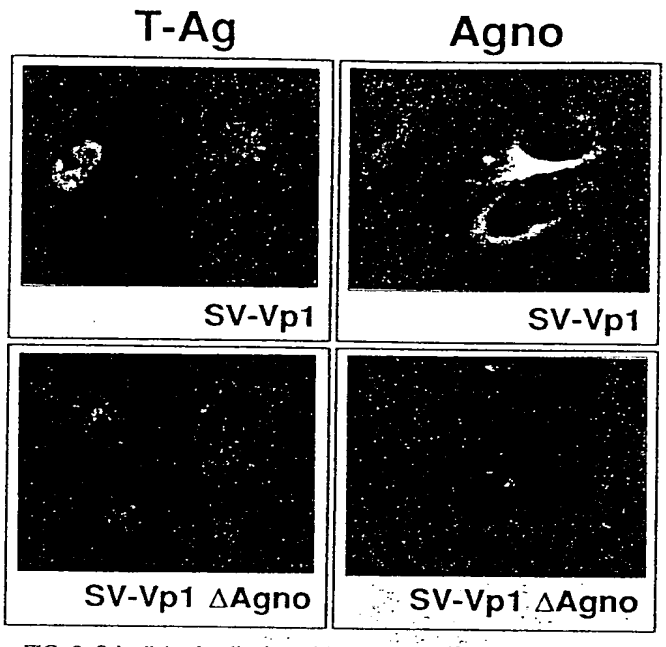

FIG. 2. Subcellular localization of large T antigen and agnoprotein. The variant viral DNAs SV-Vp1 and SV-Vp1ΔAgno were reconstituted by *Bam*HI digestion and recircularization. Culture conditions for TC7 cells, microinjection procedures, and indirect immunofluorescence have been described previously (13). DNAs (50 ng/μl) were microinjected into the nuclei of the cells, which were fixed after 24 h of incubation and reacted first with monoclonal mouse anti-SV40 T-antigen antibodies (Oncogene Science) (1:100) and rabbit antiagnoprotein antisera (1:50, kindly provided by G. Jay) and then with fluorescein isothiocyanate- and tetramethylrhodamine isothiocyanate-conjugated goat secondary immunoglobulin G (1:50), respectively. Cells were microinjected with SV-Vp1 DNA or SV-Vp1ΔAgno. The same fields of cells are shown for each variant viral DNA.

(3–18) (a deletion of residues 3 through 18), as well as a carboxyl-terminal-deletion mutant, Vp1 dl (138–362), which has a deletion of the carboxy-terminal 225 residues, were constructed (Fig. 1), and the nuclear localization capability of each mutant protein was examined (Fig. 3). For the construction of the Vp1 amino-terminal-deletion mutants, the sense primers 5′-TGCTCTAGATGAAGATGGCCGGAAGTTGTCCAGG GGCA-3′ for pSV-Vp1 dl (3–7), 5′-TGCTCTAGATGAAGA TGGCCGCAGCTCCCAAAAAACCA-3′ for pSV-Vp1 dl (3–12), and 5′-TGCTCTAGATGAAGATGGCCAAGGAACC AGTGCAAGTG-3′ for pSV-Vp1 dl (3–18) were used; the *Sac*I antisense primer was also used. For the construction of carboxyl-terminal-deletion mutants, we used a 5′-CAGGTCC ATGGTCTAGAATGAAGATGGCC-3′ sense primer and a 5′-CAAGAATTCGAGCTCGCCCAACTTGTTTATTGCA GCTTATAATGGTTACAAATAAAGCAATAGCATCACA AATTTCACAAATAAAGCATTTTTTTCAATGAGTTTTT TGTGTCCCTGAAT-3′ antisense primer. The mutants were generated by exchanging the 1,180-bp *Xba*I-*Sac*I fragment of pSV-Vp1 with the corresponding fragments bearing deletions. The structure of each construct, together with the subcellular localization of each mutant Vp1, is summarized in Fig. 3A, and typical immunostaining patterns for the mutant Vp1 proteins are shown in Fig. 3B. Although the elimination of the carboxyl 225 residues of Vp1 did not affect the nuclear localization, the elimination of the amino-terminal 5, 10, or 16 residues caused mostly cytoplasmic phenotypes (Fig. 3). These results are, in agreement with the reported observation that the SV40 Vp1 NLS is within the amino-terminal 11 residues of the protein (26).

We observed two distinct clusters of basic amino acids within the amino-terminal 20 amino acids: lysine-arginine-lysine (5 through 7) and lysine-lysine-proline-lysine (16 through 19). We have introduced a mutation(s) in the first or second cluster or both (Fig. 4) and have examined the nuclear localization capability of the mutant proteins for the following two reasons. First, many NLSs are composed of a dual signal or a bipartite signal, in which two clusters of basic residues are separated by various lengths of intervening amino acids (8, 9, 22). For example, polyomavirus large T antigen (21), *Xenopus* N1 (15), and nucleoplasmin (22) have two basic clusters, and mutation of either cluster partially impairs nuclear localization. However, simultaneous mutations of the two motifs eliminate the activity. In the case of the *Saccharomyces cerevisiae* ribosomal protein L29, either of the two clusters can target the fusion protein to the nucleus, and deletion of both clusters causes a greater loss of the nuclear localization activity (25). The two tracts of basic residues in the dual signals, therefore, are known to function in an interdependent manner. Second, the amino acid sequence of the amino terminus of SV40 Vp1 is substantially different from that of a related mouse polyomavirus Vp1 (24). Residues 5 to 19 of SV40 Vp1 have the characteristics of the bipartite NLS found in a large number of predominantly nuclear proteins, whereas polyomavirus Vp1 lacks the downstream cluster.

Plasmids bearing the amino-terminal point mutations pSV-Vp1 p6 (lysine 5 replaced with asparagine; herein designated 5N), p1 (6G), p55 (7N), p63 (16N), p26 (19N), p8 (5N6G), p33 (6G7N), p2 (17N19N), p16 (5N17N19N), p44 (6G16N19N), p46 (6G17N19N), p52 (7N16N17N), p81 (16N 17N19N), p19 (5N16N17N19N), p48 (6G16N17N19N), p39 (7N16N17N19N), p28 (5N7N16N17N19N), and p25 (5N6G 7N16N17N19N) were constructed (Fig. 1) by exchanging either the 765-bp *Xba*I-*Apa*I or the 1,179-bp *Xba*I-*Sac*I fragment of pSV-Vp1 with that of the mutation-bearing fragments. For the construction of mutants p1 to p63, the degenerate sense primers 5′-CAGGTCCAT GGTCTAGAATGAAGATGGC CCCAACAAA(A/c)(A/g)GAAA(A/c)GGAAGTTGTCCAG GGGCAGCTCCCAA(A/c)AA(A/c)CCAAA(G/c)GAAC CAGTGCAAGTGCCAAAGCTCG-3′ and the antisense primer 5′-CAAGGGCCCAACACCCTGCTC-3′ (nucleotides 2266 to 2246; designated the *Apa*I antisense primer) were used with the pSV-Vp1 template. For the p81 mutation, a 5′-CA GGTCCATGGTCTAGAATGAAGATGGCCCCAA CAAAAAGAAAAGGAAGTTGTCCAGGG-3′ sense primer and the *Sac*I antisense primer were used with the pSV-Vp1 p19 template. All point mutations are diagrammed in Fig. 4A, in which a summary of the subcellular localizations of the mutant Vp1s is given. Photographs of staining by mutants are shown in Fig. 4B.

When single point mutations were introduced into either cluster, most mutant Vp1 proteins localized to the nucleus (mutants p6, p55, p63, and p26) (Fig. 4A) except for the Vp1 mutant protein p1, in which arginine 6 was replaced with glycine and which was distributed mostly in the cytoplasm (mostly cytoplasmic phenotype). Double mutations in the upstream cluster (mutants p8 and p33) significantly affected the nuclear accumulation of the mutant proteins, while a double mutation in the downstream cluster (mutant p2) had very little effect on the localization. When three amino acids were replaced, with one mutation in the upstream cluster and two mutations in the downstream cluster (mutants p16, p44, p46, and p52), all of the mutant Vp1s except p16 showed the cytoplasmic phenotype. When all of the basic residues in the downstream cluster were

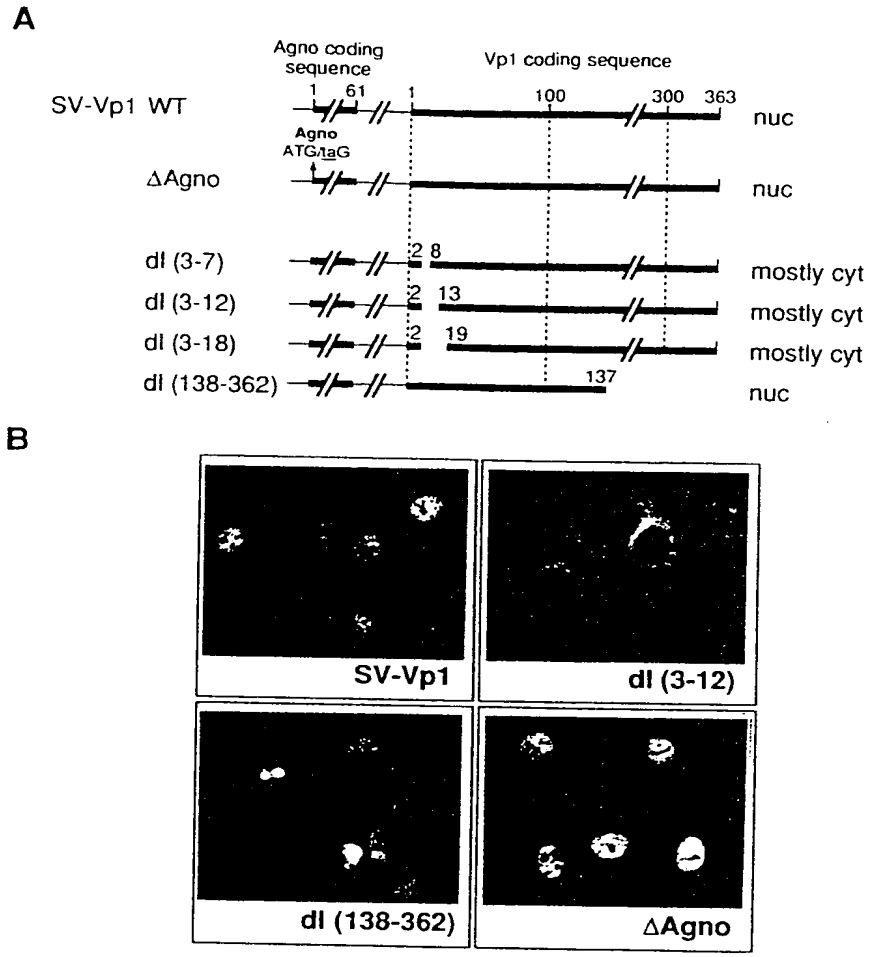

FIG. 3. Subcellular localization of Vp1 expressed from Vp1 deletion and agnoprotein mutants. Cells microinjected with each of the mutant DNAs were reacted first with the mouse anti-SV40 T antibody (1:100) and guinea pig anti-Vp1 antisera (1:50), followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (1:50) and tetramethylrhodamine isothiocyanate-conjugated goat anti-guinea pig immunoglobulin G (1:50). (A) The Vp1 coding sequence, amino acids 1 to 363, and those of the deletion mutants are shown as horizontal bars. The agnoprotein coding segment is upstream of the Vp1 gene in the SV-Vp1ΔAgno construct. The subcellular localization of each individual mutant Vp1 is at the right: nuc, distinct nuclear localization (example shown in panel B for SV-Vp1); mostly cyt, predominantly cytoplasmic localization [example shown in panel B for dl (3–12)]. (B) Immunofluorescence photographs of the cells. Cells were microinjected with DNA from SV-Vp1, dl (3–12), dl (138–362), or ΔAgno.

changed to asparagine (p81), or the p81 mutation was combined with an additional mutation in one of the upstream basic residues (p19, p48, and p39), the mutant proteins were found in the cytoplasm. Mutant proteins with more than one alteration in the upstream cluster in addition to the downstream mutations (p28 and p25) were also cytoplasmic. Within a set of mutants examined, the most influential basic residue that caused a change in the Vp1 subcellular localization was arginine 6, regardless of whether it was in the context of a single alteration (p1) or multiple alterations (p8, p33, p44, p46, p48, and p25). Lysine 5 and lysine 7 each contributed to the localization if the downstream cluster was simultaneously mutated. The simultaneous alterations of the three downstream lysines were sufficient to make Vp1 cytoplasmic.

As cysteine 10 of Vp1 has been suggested to be involved in the protein's nuclear localization (6), we next investigated the effect of the replacement of the cysteine with alanine, serine, or glycine in SV-Vp1-C10A, SV-Vp1-C10S, and SV-Vp1-C10G, respectively. For the alterations, the degenerate sense primer 5′-TGGTCTAGAATGAAGATGGCCCCAACAAAAAGA AAAGGAAGT(g/T)(G/c)TCCAGGGGCAGCTCCCAAA-3′ (lowercase letters represent mutations) and the *Apa*I antisense primer were used for PCR, and the 765-bp *Xba*I-*Apa*I fragment bearing the mutations was exchanged for that of the wild-type counterpart in pSV-Vp1. The mutation of the cysteine to alanine, serine, or glycine did not alter the nuclear localization activities of the mutant proteins (Fig. 4). It does not appear that the replacement of the cysteine with serine caused a conformational change that affected the nuclear localization capability of the conjugate protein.

In the SV-Vp1 variant viral genome, Vp1 is expressed from the late promoter, following the expression of the large T antigen (13). In an independent experiment, we placed some of the mutant Vp1s under the control of the early promoter by replacing the mutation-bearing *Xba*I-*Sac*I fragments of the pSV-Vp1s dl (3–7), p55, p8, p33, or p25 with that of pSG5/EXSB (Fig. 1). Of all mutant Vp1s examined, SG5-Vp1 dl (3–7), SG5-Vp1-p8, SG5-Vp1-p33, and SG5-Vp1-p25 showed subcellular distributions similar to those shown by SV-Vp1 dl (3–12) (Fig. 3) or SV-Vp1-p33, -p52, and -p19 (Fig. 4). A

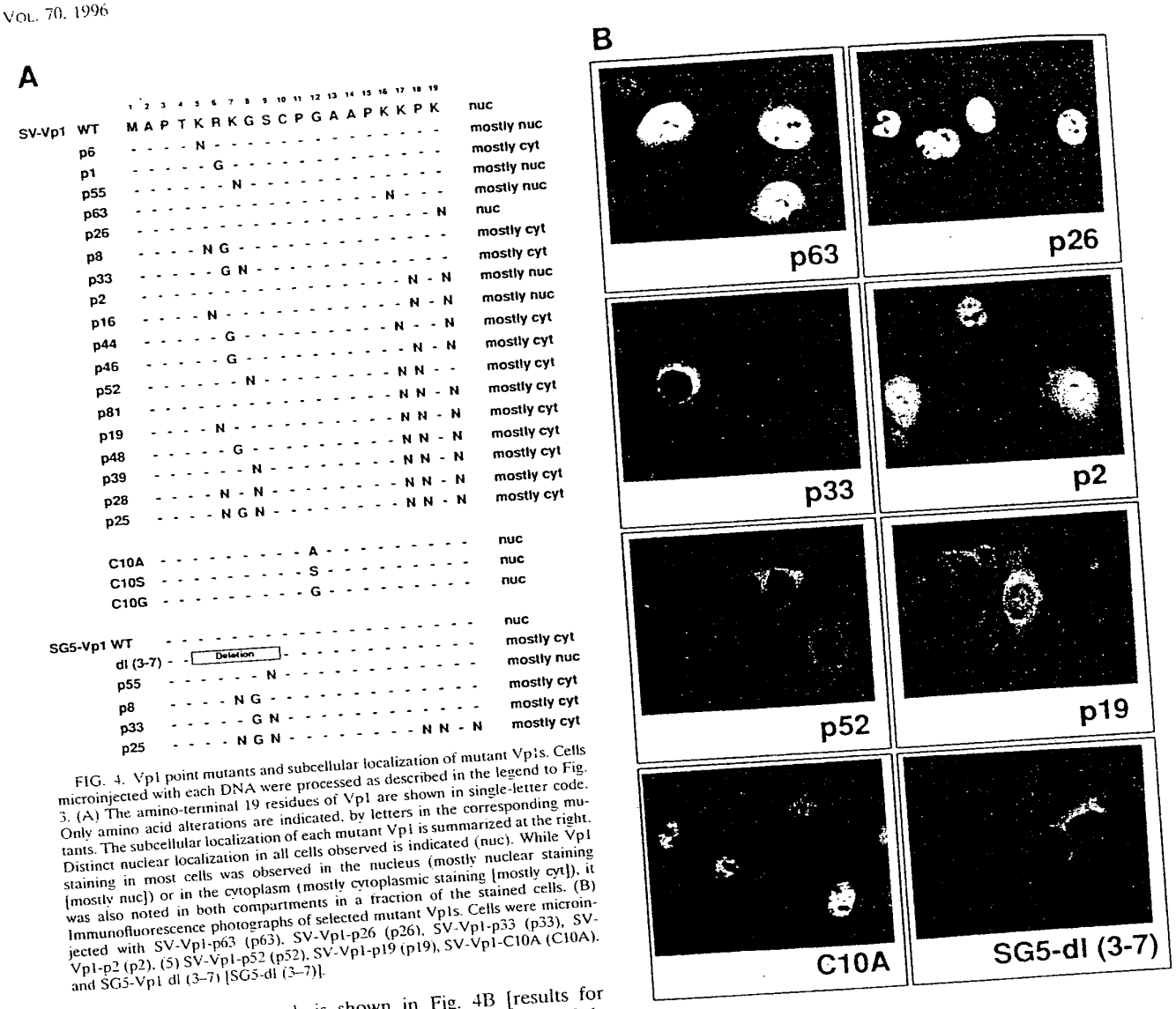

FIG. 4. Vp1 point mutants and subcellular localization of mutant Vp1s. Cells microinjected with each DNA were processed as described in the legend to Fig. 3. (A) The amino-terminal 19 residues of Vp1 are shown in single-letter code. Only amino acid alterations are indicated, by letters in the corresponding mutants. The subcellular localization of each mutant Vp1 is summarized at the right. Distinct nuclear localization in all cells observed is indicated (nuc). While Vp1 staining in most cells was observed in the nucleus (mostly nuclear staining [mostly nuc]) or in the cytoplasm (mostly cytoplasmic staining [mostly cyt]), it was also noted in both compartments in a fraction of the stained cells. (B) Immunofluorescence photographs of selected mutant Vp1s. Cells were microinjected with SV-Vp1-p63 (p63), SV-Vp1-p26 (p26), SV-Vp1-p33 (p33), SV-Vp1-p2 (p2), (5) SV-Vp1-p52 (p52), SV-Vp1-p19 (p19), SV-Vp1-C10A (C10A), and SG5-Vp1 dl (3–7) [SG5-dl (3–7)].

representative photograph is shown in Fig. 4B [results for SG5-Vp1 dl (3–7)]. The subcellular localization of SG5-Vp1-p55 was similar to those of SV-Vp1-p63 and SV-Vp1-p2, which are shown in Fig. 4B.

In summary, by using a number of SV40 Vp1 mutants, we further characterized the NLS of Vp1. The Vp1 NLS functions autonomously once the protein is synthesized in the cytoplasm, and agnoprotein does not play a role in Vp1 nuclear localization in the TC7 cell line. While the first eight amino acids of SV40 Vp1 are important for nuclear localization activity, as originally demonstrated by Wychowski et al. (26), our present findings revealed that the downstream basic residues 16 through 19 have an independent targeting activity. In SV40 Vp1, two clusters of basic residues (a cluster of lysine 5, arginine 6, and lysine 7 and a cluster of lysine 16, lysine 17, and lysine 19) are important for localization activity, the results of which contrasts with those of polyomavirus Vp1 (4).

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Association with capsid proteins promotes nuclear targeting of simian virus 40 DNA

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ABSTRACT **All animal DNA viruses except pox virus utilize the cell nucleus as the site for virus reproduction. Yet, a critical viral infection process, nuclear targeting of the viral genome, is poorly understood. The role of capsid proteins in nuclear targeting of simian virus 40 (SV40) DNA, which is assessed by the nuclear accumulation of large tumor (T) antigen, the initial sign of the infectious process, was tested by two independent approaches: antibody interception experiments and reconstitution experiments. When antibody against viral capsid protein Vp1 or Vp3 was introduced into the cytoplasm, the nuclear accumulation of T antigen was not observed in cells either infected or cytoplasmically injected with virion. Nuclearly introduced anti-Vp3 IgG also showed the inhibitory effect. In the reconstitution experiments, SV40 DNA was allowed to interact with protein components of the virus, either empty particles or histones, and the resulting complexes were tested for the capability of protein components to target the DNA to the nucleus from cytoplasm as effectively as the targeting of DNA in the mature virion. In cells injected with empty particle–DNA, but not in minichromosome-injected cells, T antigen was observed as effectively as in SV40-injected cells. These results demonstrate that SV40 capsid proteins can facilitate transport of SV40 DNA into the nucleus and indicate that Vp3, one of the capsid proteins, accompanies SV40 DNA as it enters the nucleus during virus infection.**

Various animal viruses, such as DNA tumor virus, orthomyxovirus, and retrovirus, utilize the cell nucleus as the reproductive site during some part of their life cycle. Ubiquitous as it is, the process by which these viruses target their genome to the nucleus is still little known. It has been reported that herpes simplex virus bearing one temperature-sensitive mutation within its capsid proteins is defective at delivering the viral DNA to the nucleus at nonpermissive temperatures (1, 2). Adenovirus appears to release the viral DNA into the nucleus together with the hexon, but not with many other viral proteins (3, 4). In retrovirus (5, 6), influenza virus (7–9), hepadnavirus (10), hepatitis delta virus (11), adenovirus (12), and papovavirus (13–18), many structural and virion-associated proteins have been found to harbor nuclear-targeting signals (NTSs). These results are consistent with the idea that the NTSs of viral structural proteins or/and virion-associated proteins are responsible for the nuclear entry of the viral genome.

Simian virus 40 (SV40), a member of the nonenveloped, icosahedrally symmetrical papovavirus family, is composed of three viral structural proteins, histones, and double-stranded circular DNA (19). We have recently shown that biologically functional SV40 virion enters the nucleus through the nuclear pore complex (NPC; refs. 20 and 21). Characteristic of NPC-mediated nuclear transport processes, the nuclear entry of SV40 is signal- and energy-dependent (22, 23) and sensitive to such inhibitors as wheat germ agglutinin and anti-nucleopo antibody (21). During a productive infection cycle, the vi structural proteins, Vp1, Vp2, and Vp3, localize to the nucle (24) through the NPC by virtue of their individual NTSs, whi have been identified (13–15). The karyophilic histones, sor of whose NTSs are known (25, 26), also enter the nucle through the NPC (27–29). Thus, all virion protein compone could in principle individually or collectively contribute to t nuclear entry of SV40 viral DNA. In this paper, we have test whether the nuclear entry of virion DNA is accompanied the viral structural proteins and whether it is promoted by t capsid proteins or histones.

MATERIALS AND METHODS

Cells, Microinjection, Microinfection, and Preparation Virion and Empty Particles. The cell culture conditions f TC7 cells and procedures for microinjection, microinfectic and immunocytochemistry have been described (20, 30). F the time-course study of Vp1 nuclear accumulation, about 1 SV40 virions or an equal number of SV40 empty particl (judged by protein profiles on SDS/PAGE), along with rh damin-conjugated bovine serum albumin (R-BSA), were c toplasmically injected into each of the cells located in thr marked areas on the same coverslip. For cytoplasmic antibo interception experiments, cells were cytoplasmically inject with anti-Vp1 IgG or anti-Vp3 IgG and then either cytopl mically injected or microinfected with virions. For nucle antibody interception experiments, either anti-Vp3 IgG, an β-galactosidase monoclonal antibody (anti-β-gal), or injecti solution alone was injected into the nucleus, and then virio were injected into the cytoplasm.

SV40 and empty particles were obtained essentially described (31). Empty particles were prepared either frc virus-infected cells or from virion preparations followi repeated freeze/thaw cycles and rebanding in $CsCl_2$ (20). Le than one plaque was detected from the equivalent of 2×1 physical particles, and their microinjection of empty particl did not lead to large tumor (T)-antigen expression (data n shown).

Antibodies and Immunofluorescence Microscopy. T preparation of rabbit anti-Vp1 and anti-Vp3 sera and of guin pig anti-Vp1 as well as the condition for immunofluorescen microscopy have been described (20, 32). Affinity-purifi anti-Vp1 and anti-Vp3 IgGs were prepared from polyclor sera by using nitrocellulose membrane onto which either viri

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antig NTS, nuclear-targeting signals; NPC, nuclear pore complex; BS bovine serum albumin; R-BSA, rhodamine-conjugated BSA; β-g β-galactosidase; F-dex, fluorescein-conjugated dextran.

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Vp1 or glutathione *S*-transferase (GST)-Vp3 fusion protein, respectively, had been immobilized (33). The IgG preparations used in this study were relatively free of nuclease activity, since incubation of SV40 DNA at 50 μg/ml with various concentrations (0.15–2 mg/ml), of several IgG preparations for 1 hr at room temperature or for 6 hr at 37°C did not change either the DNA mass or the distribution of the topological forms of circular DNA (data not shown).

***In Vitro* Reconstitution of Minichromosome.** Minichromosome was reconstituted *in vitro* by salt-gradient dialysis (34) and then was analyzed either by 0.7% agarose gel as described (35) or by micrococcal nuclease digestion (34).

RESULTS

Association of Structural Proteins during Nuclear Entry of Virion DNA. During SV40 infection, a virion particle adsorbs to the cell surface, enters the cytoplasm by endocytosis, and is enclosed in a vesicle (36–38). If infecting SV40 is to enter the nucleus through NPC, the NTSs must become accessible to the nuclear transport machinery. This process requires the release of the virion particle from the membrane enclosure. When the infecting virion is exposed in the cytoplasm, cytoplasmic introduction of reagents such as antibodies against viral proteins, anti-Vp1 IgG or anti-Vp3 IgG, could interfere with the nuclear entry route, thereby leading to the lack of nuclear T-antigen detection, the first sign of the SV40 gene activity. On the other hand, the antibodies would not recognize virions if they were enclosed in vesicles. Cytoplasmically injected SV40 virion is expected to be free from membrane enclosure and thus can serve as a control in the antibody interception experiments.

We tested the effect of anti-Vp1 IgG or anti-Vp3 IgG after its cytoplasmic injection on the expression of the T-antigen gene in SV40-injected or -infected cells. IgGs, confirmed to be free of nuclease activities (*Materials and Methods*), were introduced into the cytoplasm together with R-BSA, an injection marker, prior to virion injection or infection. Injection of BSA or control antibodies into the cytoplasm has no obvious deleterious effect on the expression of the T-antigen gene (21). In the injection experiments, nuclear T-antigen staining was not detected in SV40-injected cells in which anti-Vp1 IgG was cytoplasmically injected (Fig. 1*A*, arrow), as visualized by cytoplasmic rhodamin fluorescence (Fig. 1*a*, arrow), but was observed in SV40-injected cells that received no antibodies (Fig. 1 *A* and *a*, arrowhead). Identical results were obtained in cells that received or did not receive anti-Vp3 IgG (Fig. 1 *C* and *c*, arrow and arrowhead, respectively). Simultaneous injection of antibody, R-BSA, and virions into the cytoplasm gave the same results (data not shown).

In the infection experiments, the antibodies and R-BSA were injected into the cytoplasm, and cells were then infected with SV40. Results that were essentially the same as those derived from the virion injection approach were obtained either with anti-Vp1 IgG (Fig. 1 *B* and *b*) or with anti-Vp3 IgG (Fig. 1 *D* and *d*). These results indicate that the structural proteins of biologically active infecting virions become accessible to antibodies in the cytoplasm. These results are consistent with the interpretation that antibody against either of the capsid proteins, if it is present in the cytoplasm, can effectively block nuclear entry of viral DNA. There are, however, two alternative interpretations. One is that the cytoplasmic inclusion of antibodies led to the formation of large antibody-bound virion aggregates that precluded their nucleir entry. This possibility is difficult to test directly at this time. The other interpretation is that the virion dissociates in the cytoplasm, and viral proteins and DNAs enter the nucleus separately instead of associating with each other. Thus, the antibodies introduced in the cytoplasm could prevent the dissociation. Since minor coat proteins Vp2 and Vp3 are internal proteins in the virion structure (39), the observed anti-Vp3 effect would argue for this possibility. Even if virion dissociation takes place in the cytoplasm, would the structural proteins accompany the viral DNA when it enters the nucleus? This was tested by nuclear antibody interception experiments.

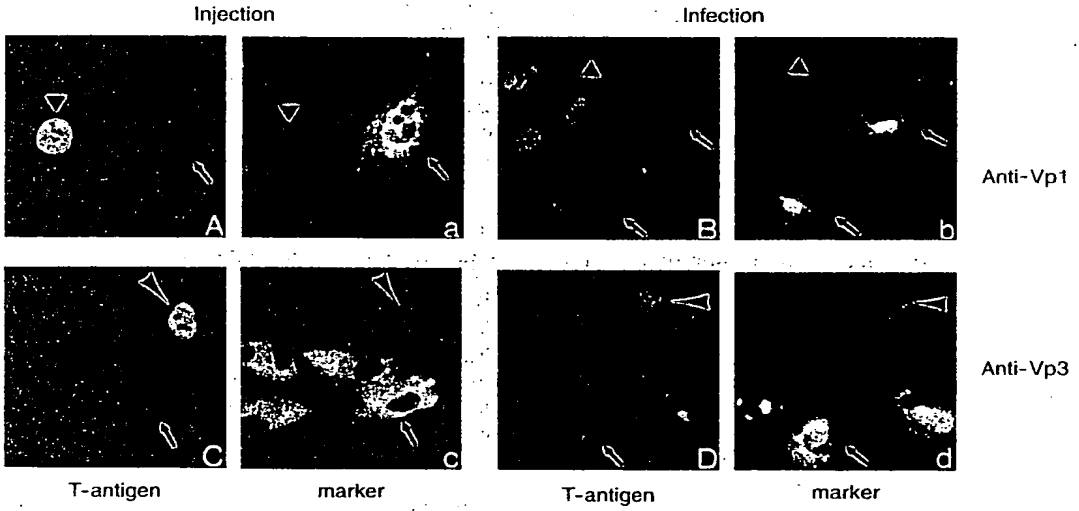

FIG. 1. Effect of cytoplasmically introduced antibodies against capsid proteins on T-antigen expression. Each pair of photographic panels (*A* and *a*, *B* and *b*, *C* and *c*, or *D* and *d*) shows the identical field of cells. Cells shown were either cytoplasmically microinjected [*Left* (*A* and *a* and *C* and *c*)] or microinfected [*Right* (*B* and *b* and *D* and *d*)] with SV40. A portion of these cells, indicated by an arrow, was cytoplasmically injected with either anti-Vp1 IgG (*Upper*) or anti-Vp3 IgG (*Lower*) at 0.5 mg/ml together with R-BSA at 1 mg/ml to mark antibody-injected cells, prior to virus injection or infection. In some cells, R-BSA was noted to accumulate at the perinuclear region. The boundary between the cytoplasm and the nucleus was ascertained by phase microscopy. All cells were harvested 6 hr after injection or 20 hr after infection and stained for T antigen. An arrowhead (the two shapes of arrowheads being equivalent) marks a virus-infected or -injected cell or group of cells that had not been injected with the antibody (plus marker) and as a result expressed T antigen. One such cell in *A*, three in *B*, one in *C*, and two in *D* can be seen to exhibit T-antigen staining and lack marker staining in *a*, *b*, *c*, and *d*. An arrow points to a virus-infected or -injected cell or group of cells that did not express T antigen as a result of having an antibody (plus marker) introduced. One such cell in *a*, two in *b*, four in *c*, and three in *d* can be seen to exhibit the cytoplasmic marker protein but do not show T-antigen staining in *A*, *B*, *C*, and *D*.

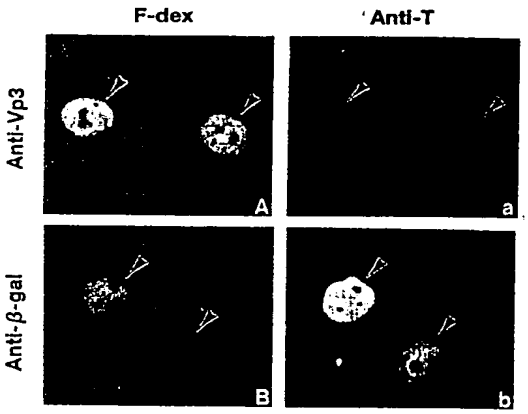

FIG. 2. Effect on T-antigen expression of nuclearly injected antibodies against structural proteins. Cells in marked areas were nuclearly injected with anti-Vp3 IgG (anti-Vp3) together with F-dex (70-kDa molecular mass) at 5 mg/ml prior to cytoplasmic injection of SV40 virions (about 10 virions per cell). As a control, monoclonal anti-β-gal was injected into cells in place of anti-Vp3 IgG. F-dex was included to mark antibody-injected cells (*A* and *B*). Cells were harvested 6 hr after virion injection, fixed in 2% paraformaldehyde as described (21), and stained for T antigen (anti-T) (*a* and *b*). An arrowhead marks a cell that had received anti-Vp3 antibody (*a*) or a cell that had received the control antibody (*b*).

When anti-Vp3 or anti-β-gal was nuclearly introduced together with the injection marker—fluorescein-dextran (F-dex)—and then ≈10 virions per cell were cytoplasmically injected, the presence of anti-Vp3 IgG in the nucleus greatly inhibited nuclear T-antigen accumulation (Fig. 2 *A* and *a*), as evidenced by only 1 of 46 and 4 of 36 injected cells (2–8%) being stained. Among 43 cells injected with anti-β-gal antibody (F-dex/anti-β-gal-positive cells, Fig. 2 *B* and *b*), 12 cells (36%) showed nuclear T-antigen staining, so the number is within the 30–40% range observed in virion-injected control cells that had received no nuclear injection treatment, in which 12 of 35 and 91 of 421 cells showed nuclear T-antigen staining. We were unable to observe inhibition of T-antigen expression by nuclear anti-Vp1 IgG interception; 15 of 56 anti-Vp1 injected cells (F-dex/anti-Vp1-positive cells) showed T-antigen staining, while 16 of 84 virion-only injected cells (F-dex-positive cells) were T-antigen positive (photographs not shown). We estimate that ≈1–5 × 10^3 and 2–10 × 10^4 antibody molecules were introduced into the nucleus and into the cytoplasm, respectively. The number of antibody molecules that could be introduced to the nucleus may not be sufficient to exert the inhibitory effect through Vp1, since 360 molecules of Vp1 are present in a single virion as opposed to about 72 molecules of Vp2 and Vp3 (24, 39). Alternatively, the result could suggest that, unlike Vp3, Vp1 was not associated with the DNA upon nuclear entry and therefore anti-Vp1 in the nucleus could not intercept T-antigen expression.

Finally, when cells were nuclearly injected with SV40 DNA along with a combination of F-dex and either anti-Vp3 IgG, anti-β-gal, or affinity-purified goat anti-rabbit IgG, all cells exhibited T-antigen expression (data not shown). Since the presence *per se* of any of these antibodies in the nucleus did not block T-antigen expression from SV40 DNA, the inhibition observed in the nuclear anti-Vp3 interception experiment was caused by the antibody's depletion of SV40 virion or of SV40 DNA–virion protein complexes that had entered the nucleus. Taking these results together with the results from the cytoplasmic antibody interception experiments, we conclude that biologically active viral DNA reaching the nucleus was in association with viral structural proteins.

Nuclear Targeting of *in Vitro* Reconstituted Protein–Viral DNA Complexes. If SV40 virion proteins promote DNA nuclear entry, protein-free viral DNA would be expected to

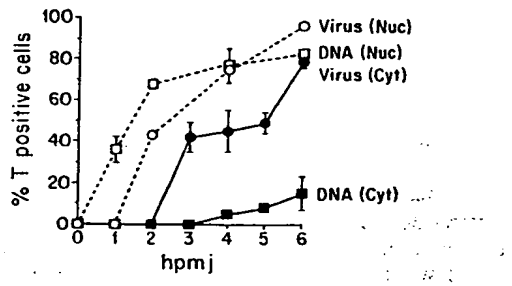

FIG. 3. Time course of nuclear accumulation of T antigen. Ce into which about 100 virions (○ and ●) or 100 SV40 DNA molecu (□ and ■) had been injected into the nucleus (Nuc) (—) or into t cytoplasm (Cyt) (—) were cultured for the indicated hours postr croinjection (hpmj), harvested, and stained for T antigen as describ (20). Cells injected with virions were doubly stained with anti-Vp1 a anti-T-antigen antibodies. The percentage of T-antigen-positive ce is the proportion of cells expressing detectable levels of T antig among Vp1-positive cells. For DNA injected cells, the percentage T-antigen-positive cells represents the proportion of the cells th expressed detectable T antigen among those marked by a fluoresce marker, F-dex or R-BSA. Vertical bars represent standard deviatic of multiple experiments.

inefficient at nuclear targeting. This difference, if any, can followed by observing the time course of nuclear accumulati of T antigen. When SV40 virus was injected into the cytoplas the percentage of T-antigen-positive cells among virio injected cells that were Vp1 positive (see Fig. 3 legen increased steadily with increasing time after injection (Fig. confirming our earlier results (20). The steady increase in t percentage of T-antigen-positive cells as well as the effectiv ness of T-antigen expression allowed virion-injected cells serve as a positive control (see below). By contrast, when t protein-free DNA was injected into the cytoplasm togeth with F-dex, T antigen was detected first 4 hr postmicroinje tion, but only in a minute fraction of the injected cells (n shown). Even after 6 hr postmicroinjection, T antigen w observed only in a small fraction of cells (Fig. 3). As a fe molecules of cytoplasmically injected SV40 DNA can bri about T-antigen expression after a long incubation time (4(the possibility that there might be selectively high nuclea activity in the cytoplasmic compartment and that this contri utes to the observed low percentage of T-antigen-positive ce in the cytoplasm is unlikely, although it cannot be entire ruled out. It is reasonable to conclude that viral DNAs virions are more rapidly and efficiently targeted to the nucle than viral DNA in protein-free form.

Inefficient Nuclear Targeting of *in Vitro* Reconstitut Minichromosomes. Histones are a component of the SV virion and form a minichromosome with the viral DNA. T viral minichromosome interacts with Vp2 and Vp3 to form internal core that the Vp1 shell encloses (39, 41). Our cyt plasmic anti-Vp3 IgG interception results could suggest th karyophilic histones (27, 28) could also become exposed up the alteration of virion structure in the cytoplasm and cou promote nuclear entry of the viral DNA. Therefore, the effe of histones on nuclear targeting of SV40 DNA was examin next. To ensure that SV40 minichromosomes contained or DNA and histones and were free from capsid proteins, t minichromosomes were reconstituted *in vitro* with SV40 DN and purified histones (34). *In vitro* reconstituted minichrom somes have been shown to retain identical properties as tho formed *in vivo* (42). Upon incubation with histones, most of t SV40 DNA became a slowly migrating species, indicating th the majority of the DNA is in nucleoprotein complex (Fig. 4 *Left Inset*, compare lanes D and C). When this reconstitut sample was digested with micrococcal nuclease, a major pr

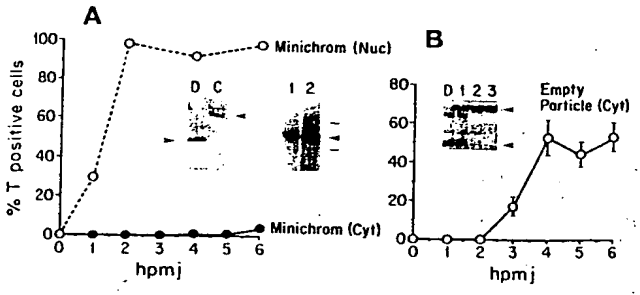

FIG. 4. Reconstitution experiments. (*A*) Effect of histones on DNA nuclear targeting. Minichromosomes were reconstituted by incubating 150 μg of SV40 DNA with 165 μg of whole histone components (Boehringer Mannheim) in 0.6 ml of 1 M NaCl/20 mM Tris·HCl, pH 8.0/1 mM EDTA/1 mM 2-mercaptoethanol and then serially dialyzing with decreasing concentrations of NaCl as described (34). The minichromosomes were injected at the DNA concentration of 50 ng/μl into either the cytoplasm (●) or the nucleus (○) of 50–100 cells per time point. The appearance of nuclear T antigen was subsequently examined as described in Fig. 3. (*A Left Inset*) Mobilities in agarose gel of protein-free covalently closed circular DNA (lane D, arrowhead) and of reconstituted minichromosomes (lane C, arrowhead; 0.5 μg per lane) are shown. One-microgram DNA samples of assembled minichromosomes were digested with 20 ng of micrococcal nuclease at 37°C for the designated period and immediately transferred to stop solution (0.2% SDS/20 mM EDTA). (*A Right Inset*) Mobility of the resulting DNA fragments in 2.5% agarose gel, after either a 30-sec digestion (lane 1) or a 15-sec digestion (lane 2) is shown. Bars at the right indicate the positions of marker fragments of 201 bp (upper bar) and 154 bp (lower bar). (*B*) Effect of empty particles on DNA targeting to the nucleus. About 1×10^{11} (0.5 μg) SV40 DNA molecules were incubated with empty particles in 15 μl of Dulbecco's PBS (Ca^{2+} and Mg^{2+} free) for 30 min at room temperature. (*Inset*) Mobility in agarose gel of 0.5 μg of SV40 DNA (lane D, bottom arrowhead, covalently closed circular DNA spieces), as well as that following incubation with 1×10^{11} (equal number) (lane 1), 1×10^{12} (lane 2), or 1×10^{13} (lane 3) empty particles is shown. The formation of high molecular weight aggregates (top arrowhead) was observed. The mixture of an equal number of DNA and empty particles, in which about half of the DNA was in protein-free DNA species (lane 1) was injected at the DNA concentration of 50 ng/μl into the cytoplasm of 50–100 cells per time point. T-antigen expression was subsequently examined as described in Fig. 3. The steady increase of the cell population expressing T antigen seen here is comparable to that seen in the cells cytoplasmically injected with virions (Fig. 3, filled circle with solid line). Abbreviations are as in Fig. 3.

tected DNA species visualized was ≈200 bp in size (Fig. 4*A Right Inset*, lanes 1 and 2), in agreement with the above observation for nucleosome formation. The reconstituted minichromosomes, when injected directly into the nucleus, were as effective as protein-free DNA or virion DNA in T-antigen expression (Figs. 3 and 4*A*). However, the minichromosomal form of SV40 DNA appeared to be insufficient for DNA nuclear targeting from the cytoplasm; T antigen was detected at 4 hr postmicroinjection, but only in a minor proportion (1%) of the cytoplasmically injected cells (Fig. 4*A*).

Empty Particles Can Promote the Nuclear Targeting of SV40 DNA. To confirm a role for capsid proteins in the nuclear targeting of viral DNA, nuclear accumulation of SV40 empty particles as well as the empty particles' effect on DNA nuclear targeting was examined. When empty particles from two different sources (*Materials and Methods*) were introduced into the cytoplasm and the subcellular distribution of Vp1 was examined, Vp1 was detected in all injected cells. When the concentration of the empty particles was raised 10–50 times, Vp3 staining was observed and was identical to Vp1 staining (data not shown). The kinetics of nuclear uptake of structural proteins from intact virions and from empty particles was indistinguishable.

When the viral DNA was incubated with empty particles and mobility alteration in agarose matrix was examined, most of the SV40 DNA was found just below the loading wells (Fig. 4*B*, compare lane D with lanes 1, 2, and 3), presumably indicating the formation of high molecular weight aggregates. When the incubation mixture (1:1) was injected into the cytoplasm and the subcellular localization of empty particle-derived Vp1 and T-antigen expression was followed, the Vp1 staining was found primarily in the nucleus (data not shown). Nuclear T-antigen staining began to appear 3 hr postmicroinjection among Vp1-positive cells, and the fraction of T-positive cells increased thereafter (Fig. 4*B*). The time course of T-antigen expression was essentially the same as that observed for virion-injected cells (Fig. 3). Thus, empty particles were effective in targeting DNA into the nucleus from cytoplasm.

The results presented above suggest that the structural proteins of SV40 are in association with viral DNA as it enters the nucleus during normal SV40 infection. Furthermore, the virion proteins promote nuclear targeting of the viral DNA.

DISCUSSION

We demonstrated that one of the capsid proteins of SV40 is important for the nuclear targeting of the viral DNA, using two independent approaches—antibody interception experiments and reconstitution experiments. The results presented here and reported previously (21) indicate that the DNA of infecting SV40 remains associated at least with Vp3 as it enters the nucleus through the NPC and that the viral structural proteins are the major promoter for the nuclear entry of the viral genome. These results also provide evidence for a release of virions from endocytotic vesicles during infection or an equivalent phenomenon that exposes virion structural proteins to the cytoplasm prior to virion nuclear entry. This result is the first demonstration of protein-directed nuclear targeting of double stranded DNA in mammalian cells.

What are the important domains in the virion that direct DNA to the nucleus? The viral proteins require independent karyophilic property and DNA interactive capability that are represented as an NTS and a DNA-binding domain, respectively. All SV40 structural proteins are sequence-nonspecific DNA-binding proteins (33, 43) and carry an individual NTS (13–15). The observation that some mutants where Vp2 and Vp3 are defective in DNA binding are nonviable (44) emphasizes the importance of the binding domain in virus infection. Although the DNA-binding domain of SV40 Vp1 is not mapped, that of polyomavirus Vp1 is within the Vp1-NTS itself (17, 45), and therefore Vp1–DNA binding could potentially mask the functioning of the NTS. Unlike SV40 Vp2 and Vp3 (33), the polyomavirus counterpart proteins do not bind DNA (46), implying that different structural proteins play a role in the DNA nuclear targeting of the two related viruses. How the antibodies introduced into the cytoplasm act to intercept T-antigen expression is not known at this time. The mechanisms of how structural proteins play roles in DNA nuclear targeting, as well as which of the SV40 structural proteins effectively targets DNA, could be determined by using wild-type or mutant proteins defective in nuclear targeting and/or in DNA binding when these two signals of SV40 Vp1 are precisely mapped.

In the SV40 virion, the NTSs of the viral proteins do not appear to be exposed at the virion surface. The Vp1 NTSs are inside the virion structure, proximal to the central core structure formed by the interaction of an SV40 DNA minichromosome with the minor coat proteins Vp2 and Vp3 (39). How the Vp2 and Vp3 molecules and their NTSs and DNA-binding domains, all of which are within the virion, are situated in the virion is not known. Based on the presence of electron-dense, virion-like structures seen in the nucleus during the infection, virus uncoating has been suggested to take place in the nucleus

(36, 47). When an intact virus passes through the NPC, the interior signals will not be recognized. Our result that nuclear T antigen was not detected when anti-Vp3 IgG was present in the cytoplasm argues for the idea that structurally altered, rather than intact, SV40 enters the nucleus and that some of the signals become accessible to the transport machinery.

Why histones that are karyophilic and can bind DNA could not target DNA in a minichromosomal form to the nucleus is intriguing. The NTS of H2B, the one defined signal of the four histones (26), lies in amino acids 20–33 of the protein but is not within residues visualized by the x-ray crystallography of a nucleosome core (48). The result could suggest that the targeting signals became inaccessible when minichromosomes were formed. In regard to the interaction of the empty particle with DNA, the observation that multiple empty particles were required to form DNA-empty aggregates (Fig. 4*B*) suggests that only a few binding domains of a particle are available for the interaction. The interaction of DNA with capsid proteins in the empty particle might be different from that in the virion. The fact that the capsid proteins were able to target DNA to the nucleus indicates that it is not necessary to maintain an intact structure to observe capsid protein-assisted nuclear targeting of DNA. Since capsid proteins of SV40 bind DNA in a sequence-nonspecific manner (33), the result could also imply that the capsid is potentially capable of targeting heterologous DNA to the nucleus. The importance of NTS and DNA-binding activities in the formation of plant crown gall tumor, which is caused by the transfer of single-stranded T-DNA from *Agrobacterium* into plant cell nucleus, have recently been shown (49–51). Conceivably, the nuclear entry of nucleic acids in general is facilitated by interaction of the nucleic acids with karyophilic proteins. For nucleus-utilizing viruses, the association of NTS-harboring virion protein components with the viral genome could be a general mechanism for targeting the genome to the nucleus.

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Principles of Molecular Virology

2nd Edition

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Class II: Single-stranded DNA

Both the autonomous and the helper virus-dependent parvoviruses are highly reliant on external assistance for gene expression and genome replication. This is presumably because the very small size of their genomes does not permit them to encode the necessary biochemical apparatus. Thus, they appear to have evolved an extreme form of parasitism, utilizing the normal functions present in the nucleus of their host cells for both expression and replication (Figure 5.5). The members of the replication-defective *Dependovirus* genus of the parvovirus family are entirely dependent on adenovirus or herpesvirus superinfection for the provision of further helper functions essential for their replication. The adenovirus genes required as helpers are the early, transcriptional regulatory genes such as E1A rather than late structural genes, but it has been shown that treatment of cells with u.v., cycloheximide, or some carcinogens can replace the requirement for helper viruses. Therefore, the help required appears to be for a

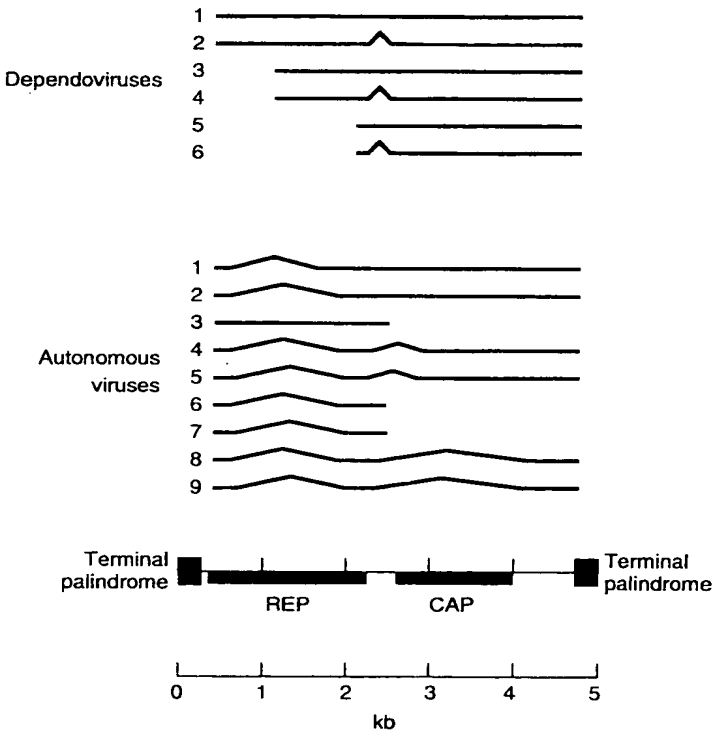

Figure 5.5 Transcription of parvovirus genomes is h avily dependent on host cell factors and results in the synthesis of a seri s of splic d, sub-genomic mRNAs which encode two proteins, rep, which is involved in genom replication and cap, the capsid protein (see text).

modification of the cellular environment (probably affecting transcription of the defective parvovirus genome) rather than for a specific virus protein.

The geminiviruses of plants also fall into this class of genome structures (Figure 3.15). The expression of their genomes is quite different from that of parvoviruses, but nevertheless still relies heavily on host cell functions. There are open reading frames in both orientations in the virus DNA, which means that both (+) and (−)sense strands are transcribed during infection. The mechanisms involved in control of gene expression have not been fully investigated, but at least some geminiviruses (subgroup I) may use splicing.

Class III: Double-stranded RNA

All viruses with RNA genomes differ fundamentally from their host cells, which of course possess double-stranded DNA genomes. Therefore, although each virus must be biochemically 'compatible' with its host cell, there are fundamental differences in the mechanisms of virus gene expression from those of the host cell. Reoviruses have multipartite genomes (see Chapter 3) and replicate in the cytoplasm of the host cell. Characteristically for viruses with segmented RNA genomes, a separate monocistronic mRNA is produced from each segment (Figure 5.6).

Early in infection, transcription of the d/s RNA genome segments by virus-specific transcriptase activity occurs inside partially uncoated sub-viral particles. At least seven enzymatic activities are present in reovirus particles to carry out this process, although these are not necessarily all separate peptides (Table 5.3, Figure 2.12). This primary transcription results in capped transcripts that are not polyadenylated and which leave the virus core to be translated in the cytoplasm. The various genome segments are transcribed/translated at different frequencies, which is perhaps the main advantage of a segmented genome. RNA is transcribed conservatively, i.e. only (−)sense strands are used, resulting in synthesis of (+)sense mRNAs, which are capped inside the core (all this occurs without *de novo* protein synthesis). Secondary transcription occurs later in infection inside new particles produced in infected cells and results in uncapped, non-polyadenylated transcripts. The genome is replicated in a conservative fashion (c.f. semi-conservative DNA replication). An excess of (+)sense strands are produced which serve as late mRNAs and as template for (−)sense strand synthesis (i.e. each (−) strand leads to many (+) strands, not one-for-one as in semi-conservative replication).

Class IV: Single-stranded (+)sense RNA

This type of genome occurs in many animal viruses and plant viruses (Appendix 1). In terms of both the number of different families and the number of individual

The cryptic life style of adeno-associated virus

Kenneth I. Berns and R. Michael Linden

Summary

Although 80-90% of adults are seropositive for antibodies against the human parvovirus adeno-associated virus (AAV), infection has not been associated with either symptoms or disease. In cell culture, AAV infection is not productive unless there is a coinfection with a helper virus, either adenovirus or any type of herpes virus; in the absence of a helper virus coinfection the viral genome is integrated into the genome, usually at a specific site on chromosome 19q13.3-qter. The integrated genome can be activated and rescued by subsequent super infection by a helper virus. The high frequency of site-specific integration by AAV and the lack of associated disease have encouraged the use of AAV as a vector for gene therapy. This review will focus on the molecular mechanisms involved in the establishment of, and rescue from, the latent state and their relevance to use of AAV as a vector.

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Introduction

The members of the family *Parvoviridae* are small, single-stranded DNA viruses that infect both vertebrates and invertebrates[1]. The virion is icosahedral with a diameter of 20-30 nm, is non-enveloped, and contains a DNA genome of about 5 kb. The adeno-associated viruses (AAV) constitute the *Dependovirus* genus, so-called because of the usual requirement for a helper virus coinfection to enable productive AAV infection to occur. AAV specific for species ranging from chickens to man have been isolated. Thus, the virus is widespread and is notable because infection has never been associated with disease[2].

The helper functions required for productive infection in cell culture have been best characterized for adenovirus (Ad) coinfection. With the exception of E2B and E3, all of the Ad early functions (E1A, E1B, E2A, E4) contribute to the helper effect in cell culture[3,4]. All of these affect gene expression, both cellular and viral. Of note is the absence of helper effects by the Ad DNA polymerase and terminal protein[5-7]. However, a different set of helper functions is supplied when Herpes simplex virus (HSV) is the helper virus. In this case, HSV enzymes directly involved in viral DNA synthesis have been implicated as helper functions[8,9]. Because of the requirement for a helper virus coinfection, AAV has been considered to be a defective virus. However, because of the variability of functions supplied by different helper viruses and because in each case some of the functions supplied have a general effect on gene expression, the notion of defectiveness has been challenged. A current hypothesis is that if AAV infects a healthy cell, the genome is programmed to repress the gene expression required for productive infection so that the viral genome can integrate into the host genome to establish a latent infection. Subsequent exposure of the cell to conditions which activate stress response genes also causes activation of the AAV genome, with rescue and replication of the integrated DNA and production of progeny virions (i.e. a productive infection). The hypothesis has been supported by the observation, by several laboratories, that exposure of several cell lines to genotoxic stimuli (e.g. UV irradiation or chemical carcinogens) makes the cells permissive for productive AAV infection in the absence of helper virus[10,11].

The purpose of this review is to discuss the molecular mechanisms thought to be involved in the establishment, maintenance and rescue of AAV from the latent state.

Genetics

The 4680 nucleotide sequence of the AAV genome has been determined[12]. There are two large open reading frames (orf) (Fig. 1). The orf in the 5′ half encodes four non-structural, regulatory proteins with overlapping amino

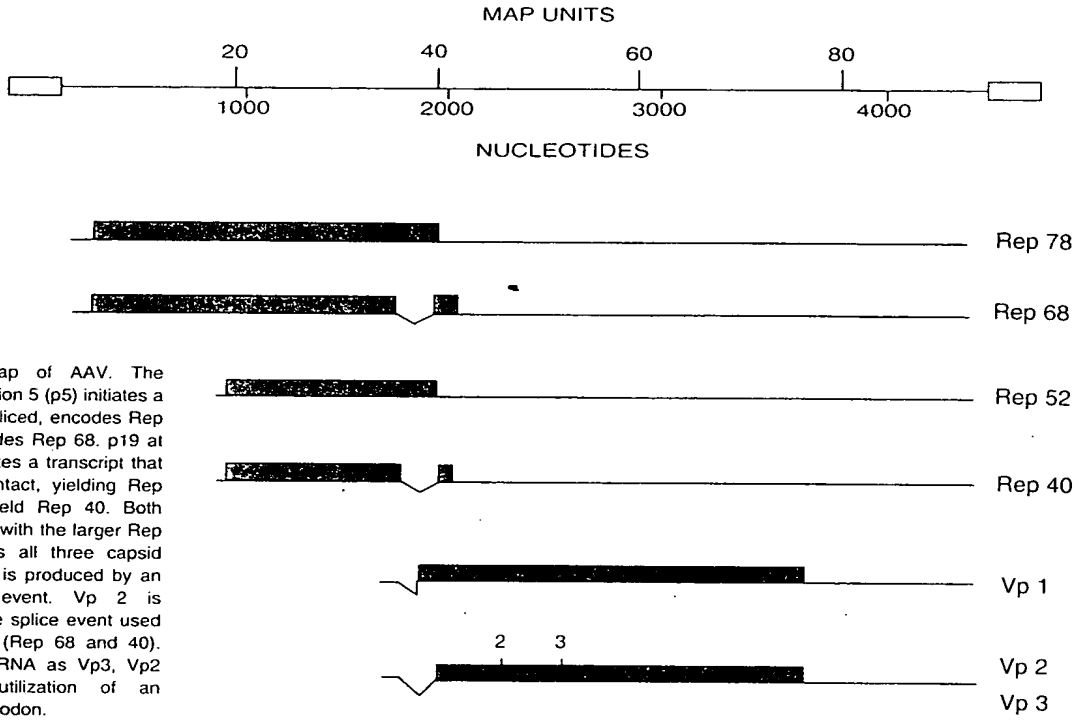

Fig. 1. Genetic map of AAV. The promoter at map position 5 (p5) initiates a transcript which, unspliced, encodes Rep 78 and spliced, encodes Rep 68. p19 at map position 19 initiates a transcript that is either translated intact, yielding Rep 52, or spliced, to yield Rep 40. Both products are in-frame with the larger Rep proteins. p40 initiates all three capsid mRNA species. Vp 1 is produced by an alternative splicing event. Vp 2 is produced by the same splice event used for the Rep proteins (Rep 68 and 40). Utilizing the same mRNA as Vp3, Vp2 results from the utilization of an alternative ACG start codon.

acid sequences, and that in the 3′ half encodes three structural proteins, again with overlapping amino acid sequences. The 5′ orf has been dubbed the *rep* gene, because almost every nonsense mutation in the orf inhibits DNA replication[13-15]. There are two transcripts of the *rep* orf with promoters at map positions (mp) 5 and 19. Both spliced and unspliced forms of each transcript are translated to yield four Rep proteins, Reps 78, 68, 52 and 40. The two larger Rep proteins have been implicated in regulation of gene expression[16,17] and DNA replication[18]. Their probable effects on site-specific integration of the DNA and rescue of the DNA from the integrated state are less well-documented. Function(s) of the two smaller proteins are less certain; in one instance they have been implicated in the packaging of the linear single strands[19]. Rep 68/78 binds to specific sites on the AAV genome (the inverted terminal repeat [itr] and in each of the three promoters), makes a site-specific nick in the genome (see below) and can function as an ATPase and helicase[20]. One Rep domain has sequences related to the ATP binding regions in the SV40 and polyoma large T antigens[21,22].

There are three viral coat proteins, Vp 1-3, which range in size from 85-65 kD. The amino acid sequences overlap, the larger proteins being extended at the amino termini. All three are translated from a transcript whose promoter is at map position 40[23]. There are alternatively spliced mRNAs; Vp 1 is translated from the minor species and the other two from the more abundant mRNA. Vp 3 accounts for about 80% of the protein mass of the virion and is translated from the first in-frame initiator codon in the major mRNA species. Vp 2 is initiated from an upstream, in-frame ACG codon[24], which presumably accounts for its minority status. Any special functions to be attributed to the minority species remain to be determined. The coat proteins may well be modified, as each can be fractionated into two or three differently migrating species on SDS-polyacrylamide gels.

The major special feature of the genome is the presence of palindromic, inverted terminal repeats. The itr is 145 nucleotides, of which the first 125 constitute an overall palindrome interrupted by two smaller 21 nucleotide palindromes immediately flanking the axis of symmetry of the overall palindrome. When folded on itself to maximize potential base pairing, the overall palindrome forms a T-shaped structure (Fig. 2) in which there are only seven unpaired bases. With a single exception, all of the base pairs in the short internal palindromes are GC pairs; all of the unpaired bases are As or Ts. Genetic experiments suggest that for some of the steps in the AAV life cycle, the potential conformation of the itr may be more important than the actual sequence. Like the Rep proteins, the itr is

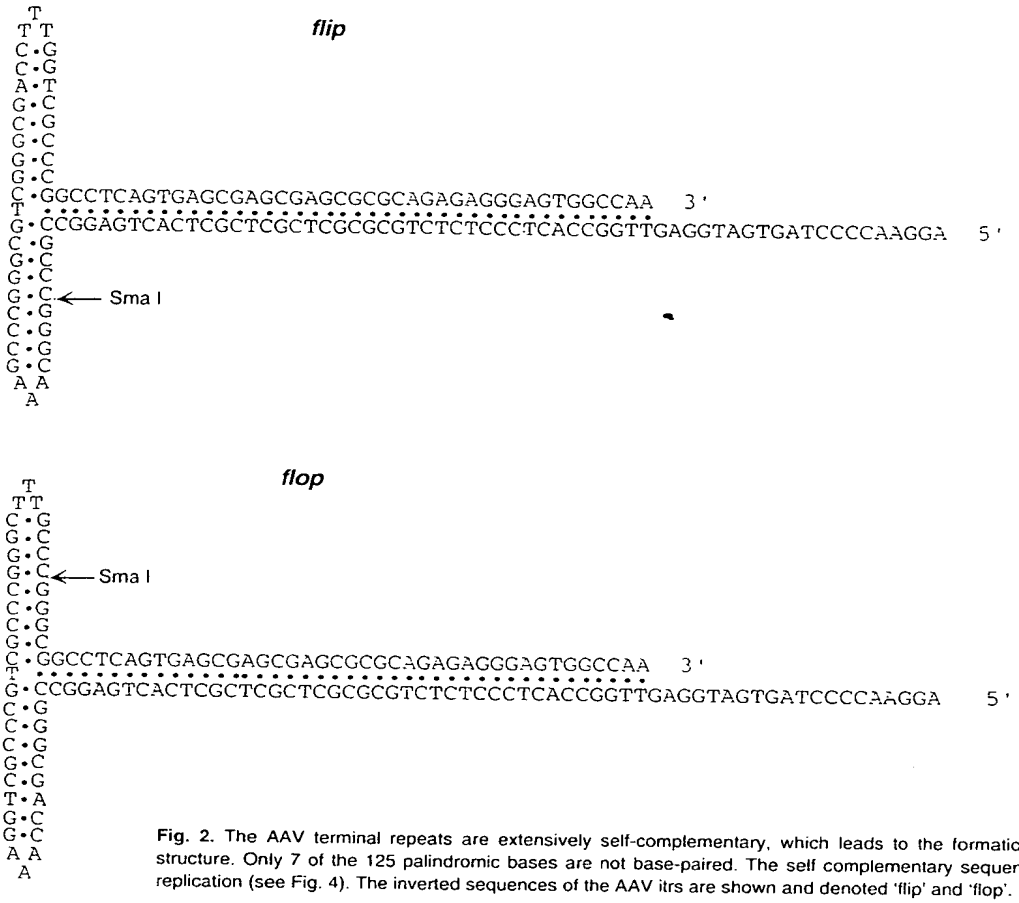

Fig. 2. The AAV terminal repeats are extensively self-complementary, which leads to the formation of a stable hairpin structure. Only 7 of the 125 palindromic bases are not base-paired. The self complementary sequence is involved during replication (see Fig. 4). The inverted sequences of the AAV itrs are shown and denoted 'flip' and 'flop'.

nvolved in every step of the AAV life cycle: regulation of ene expression, regulation of DNA replication, site-pecific integration and rescue of the genome from the ntegrated state[25-27].

roductive infection

n the presence of a helper virus, AAV undergoes produc-ive infection (Fig. 3). The virion penetrates to the nucleus f the cell and the genome is uncoated. If the cell is per-nissive for AAV replication, full gene expression occurs; if ot, gene expression is repressed and integration of the iral DNA to establish latency occurs. Under permissive onditions the first detectable viral product is RNA, but the mplate for transcription is uncertain; is it the single-tranded genome or a duplex derivative? AAV is unusual that half the virions contain the plus DNA strand and half e minus strand, so that it is conceivable that complemen-ry strands could anneal to create a double-stranded NA template for transcription. For several reasons this ossibility seems unlikely; the kinetics of infection are single hit, and other parvoviruses that package only the minus DNA strand face the same question. Alternatively, a small amount of DNA synthesis could occur to produce duplex DNA. One round of synthesis might not be detectable. This possibility seems likely, but AAV transcription has been noted under conditions that were not permissive for DNA replication. AAV Rep 68/78 is a transactivator for transcription from all three AAV promoters (p5, p19 and p40; see above). As noted above, Ad early functions are required too, especially E1A. In their absence, Rep 68/78 is a negative regulator of AAV gene expression. Recently, binding sites for Rep have been described for all three promoters and there is evidence of interaction among the different promoter regions during transcription[28,29]. There are binding sites for the cellular YY1 factor both upstream and downstream from the p5 promoter[30]. Whether the upstream site functions to inhibit transcription in the absence of E1A is in question, but the downstream site appears to act in a positive manner (i.e. in transactivation). The AAV genome contains two additional regulatory regions. The itr has been demon-

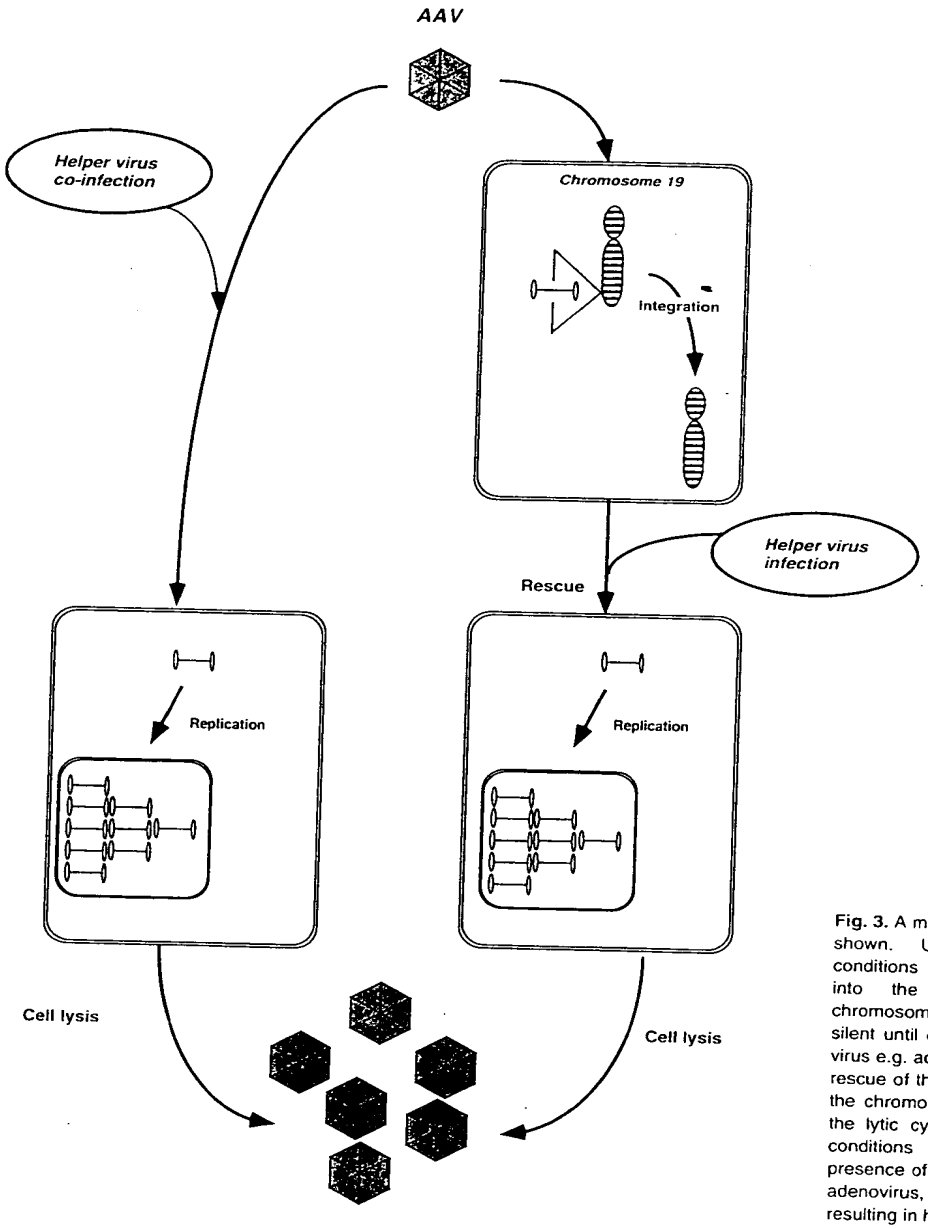

Fig. 3. A model for AAV infection is shown. Under non-permissive conditions (right), AAV integrates into the q-arm of human chromosome 19, where it remains silent until challenged by a helper virus e.g. adenovirus. This leads to rescue of the integrated virus from the chromosome and induction of the lytic cycle. Under permissive conditions (left), i.e. in the presence of a helper virus such as adenovirus, AAV replicates, resulting in host cell lysis.

strated to function as an enhancer. Of more novel aspect is a region in the middle of the *rep* orf (between map positions 10 and 37); this region down-regulates the expression of *rep* at a step after the initiation of transcription. As a consequence, the accumulation of *rep* transcripts is only about 10-20% those of p40 transcripts[17].

The linear single strand genome is replicated by a single strand displacement mechanism (Fig. 4) of the same type as that used for Ad DNA replication[31]. The palindromic itr functions as a DNA primer to produce a duplex replicative intermediate, which is covalently crosslinked at one end[32]. This hairpin is nicked on the progeny strand between nucleotides 124 and 125; a 3′ OH at the nick site serves as a primer and the displaced hairpin as a template for synthesis to restore the 3′ end of the parental strand. The newly restored end can be strand-separated, and the 3′ terminus can fold over to prime a new round of synthesis. Duplex dimers (and higher forms) can be produced if the original hairpin crosslink is not resolved; when the 3′ end of the growing strand reaches the 5′ end of the tem-

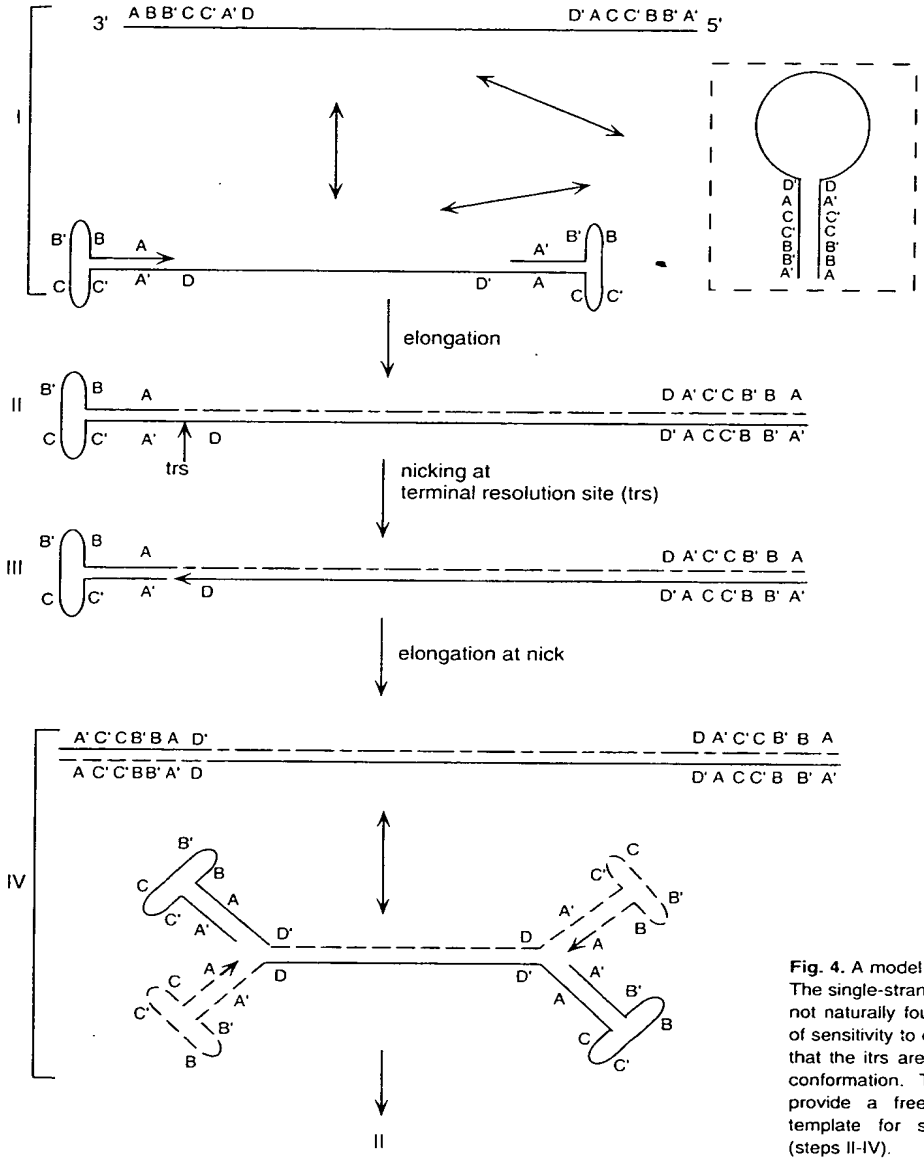

Fig. 4. A model for AAV replication is shown. The single-stranded isomer shown in step I is not naturally found, as indicated by the lack of sensitivity to endonuclease S1, suggesting that the itrs are usually in a partially duplex conformation. The hairpinned isomer can provide a free 3′ hydroxyl group as a template for self-primed DNA replication (steps II-IV).

plate strand, the 3′ end can fold on itself and synthesize a new strand equivalent, now using the first daughter strand as the template. The initial step of the first round of replication may be a simple hairpinning of the 3′ end to serve as a primer, or it may be the formation of a single-stranded circle stabilized by H-bonding between the itrs at the ends of the genome (Fig. 4, dashed box in step I). With the latter model the duplex structure of the end is similar to the ends of the duplex replicative intermediates in Fig. 4, step IV, so that the mechanism of initiation could be identical.

Several *in vitro* assays for DNA replication have been developed, allowing the beginning of investigations towards an understanding of the biochemistry of the process. One assay uses a plasmid construct containing either the itr or linear duplex AAV DNA as the template[33,34]. In the former case, the AAV insert is rescued and replicated. The reaction mixture contains an extract from cells coinfected by AAV and Ad. Extracts from uninfected cells or cells infected with either AAV or Ad do not work. However, if any of these is supplemented with recombinant Rep 68 or Rep 78, replication occurs[34]. Thus, at this level the only non-cellular component identifiable in the replication reaction is Rep 68/78 and there is no apparent need for any specific Ad gene products. However, data obtained with the second reported

in vitro assay does suggest that there is a component in Ad-infected cells that is not present in uninfected cells[35]. In this assay the template is linear duplex DNA covalently crosslinked by the terminal hairpins. This template structure is that of a hypothesized replicative intermediate in the current model (except for the second hairpinned end). Even with the addition of recombinant Rep, an Ad-infected cell extract is required, although there may be a minimal amount of replication with an uninfected cell extract. Furthermore, when a non-crosslinked, linear, duplex template is used, use of the extract from Ad-infected cells seems to result in more consistent full-length copying of the original template strand. Use of the uninfected cell extract permits folding back of the elongating strand with consequent template strand switching, leading to synthesis of defective DNAs. In both assays only a small minority of the duplex products have both strands newly synthesized and there is very little evidence for reinitiation of a second round of DNA synthesis. No evidence for Okazaki fragments has been found[36]; thus, lagging strand synthesis seems very unlikely. Several cellular replication components have been identified (N. Muzyczka, personal communication); these include a single-stranded DNA binding protein (RFA) and the protein (RFC) that interacts with DNA polymerase delta. Thus, DNA polymerase delta is probably involved and possibly DNA polymerase alpha (there is some suggestion *in vivo* that when HSV is the helper, the HSV DNA polymerase may be used). Finally, there is evidence for a component which is present in fraction IIA (in the standard mammalian cell fractionation protocol[37]) and which does not correspond to one of the DNA polymerases in the fraction. Use of antibodies against mammalian topoisomerases does not inhibit the DNA replication assay (N. Muzyczka, personal communication).

AAV capsids self-assemble in insect cells infected with recombinant baculoviruses containing the structural genes[38]. Thus, it seems possible that the capsids are preformed and that they interact with replicative intermediates to sequester single strands for encapsidation. In addition to the capsid genes, there is one report implicating Rep 52/40 in this process[39]. In cell culture the progeny virions are cell-associated and found in crystalline arrays in the nucleus; the mechanism of release has not yet been well defined. Replication is associated with cell death, but the specific relationship to AAV is uncertain because the helper Ad and herpes viruses cause lytic infections.

Latent infection

AAV latency was discovered by Hoggan *et al.*[40], who found that 20% of primary African green monkey kidney cell lots and 1-2% of primary human embryonic kidney cell lots would produce AAV upon infection of the cells with purified Ad. The latent infection was readily established in continuous lines of human cells in culture by infection with AAV at relatively high multiplicity of infection. (100-250 infectious units/cell) in the absence of helper virus[41]. In the original experiments, 30% of the cells cloned after 44 passages produced AAV upon challenge with Ad. Early analysis of the viral DNA in clones of latently infected cells showed that the viral genome was integrated into cellular DNA[25,42]. Subsequent studies have led to the following model for the establishment and maintenance of latent infection (Fig. 3). Under non-permissive conditions (i.e. absence of helper virus coinfection) the AAV particle enters the cell and penetrates to the nucleus where the DNA is uncoated. A small amount of Rep protein is synthesized, which represses further AAV gene expression. Indirect evidence also suggests that Rep can inhibit viral DNA replication under non-permissive conditions[18]. The consequence of the inhibition is to favor integration of the AAV genome into the cellular genome in a manner which is unique. Any exogenous DNA, no matter the manner in which it enters the mammalian cell, will recombine with cellular DNA. Recombination is almost invariably non-homologous and occurs in a manner which appears to be random (it is possible to demonstrate that retrovirus integration into actively transcribed areas of the genome is favored). However, in continuous lines of human cells, 70-100% of the time, AAV integrates at a specific site on chromosome 19q13.3-qter[26,27,43-45]. The only homology between the integrated AAV DNA and the flanking cellular sequences is a 1-5 base overlap at the junction. When integrated copies of the AAV genome were cloned out, it was apparent that integration was associated with rearrangements of both viral and flanking cellular sequences. The ability to rescue wild-type AAV from such latently infected cell clones was apparently the consequence of the fact that in almost every case the integrated AAV sequences were present as a linear tandem of several copies of the AAV genome; presumably at least one of the copies was present in the wild-type form. In approximately half the cases, the itr was at the junction with cellular sequences, although in every case some of the terminal sequences had been deleted. The rest of the junctions occurred near the p5 promoter (R. J. Samulski, personal communication).

A major question was whether the specific sequence of the integration site or its location on chromosome 19q was the determining factor in the site specificity . To address this, the preintegration site was cloned and inserted into an Epstein-Barr Virus-based shuttle vector, which was established in a line of human cells[46]. When these cells

were infected by AAV, the viral genome integrated into the episome, but did not integrate into episomes lacking critical portions of the preintegration sequence. Therefore, the primary sequence of the preintegration site directs site-specific integration. The critical sequences have been mapped to a 510-base sequence. Of particular interest within this region are three potential signal sequences. All three of the signals are present at the left end of the AAV genome, in approximately the same spatial orientation as observed in the preintegration sequence. The first is a dodecamer $(GCTC)_3$, which is a binding site for Rep 68/78. The second is the terminal resolution site (trs) nicked by Rep. Finally there is a sequence that shares homology with a recombination enhancer found in yeast, the M26 motif. The Rep-binding site and trs are also present at the right end of the genome in the itr, but the possible recombination enhancer is not. Although there is a specific site of integration on chromosome 19, the mapped junctions between viral DNA and cellular sequences do not map to identical positions in the nucleotide sequence in different, independently derived clones. There is a variability of several hundred bases. The previously mapped junctions have been about 500-700 bases downstream from the 510-base sequence of the preintegration site, which has been determined to direct site-specific integration. Thus, the recognition site that determines site specificity apparently acts at a relatively short distance from the actual sites of recombination.

For a cryptic virus such as AAV, latent infection might be expected to have little, if any, effect upon the host cell. However, the viral genome is inserted into a sequence that is expressed in several tissues and Rep is a powerful effector of gene expression. Indeed, no phenotypic effects as a consequence of latent infection were noted in the original primary cell lots positive for the virus or in the clones in latently infected human cells. Viability and cell division were not affected. However, as more detailed examination of expression from specific genes has been carried out, there have been observations of both up- and down-regulation[47-49]. In a series of experiments in which Chinese hamster embryo cells were latently infected by DNA, it was noted that latency was associated with increased sensitivity to UV-irradiation and the ability of UV-irradiation to induce gene amplification was decreased[50]. Although such effects could be the result of insertional mutagenesis, there is a strong suspicion that the effects are the consequence of *rep* gene expression (although in the case cited immediately above, there was not a strict correlation between the amount of Rep detected and the magnitude of the phenotypic effect). Rep proteins are powerful effectors of gene expression and under conditions where Rep expression is not repressed, a variety of cytotoxic phenomena have been noted. The key issue is probably that under physiological conditions of latent infection, Rep proteins autoregulate their own expression and, thus, significant cytotoxic effects are avoided.

AAV as a vector for gene therapy

AAV has several biological properties that would be useful for a vector for gene therapy. These include (1) lack of association with disease; (2) the ability to latently infect a high fraction of exposed cells (in culture); (3) a minimal number of viral antigens to induce a host immune response; (4) the possible (but not conclusively shown) ability to latently infect non-dividing cells; (5) the possible advantage of site-specific integration and (6) the ability to latently infect a broad range of human cell types[51-54]. As various problems associated with the use of other vector systems have become manifest, there has been an increased effort to develop AAV as a vector. The first limitation inherent in the use of an AAV vector is the maximum size of the insert, which is about 4.5 kb. With an insert of this size the only AAV sequences in the DNA are the itrs at each end. In practice most vectors have been of this general type. A good example is psub201[55,56]. This vector has the 3′ 190 bases of AAV (from an *Xba*I site through the right itr) also present at the 5′ end of the AAV insert; digestion with *Xba*I drops out the unique AAV sequences, which can be replaced by the desired gene. To produce the viral vector, the modified psub201 plasmid is co-transfected into a cell with a helper plasmid containing the unique AAV sequences (deleted above from the vector construct) inserted between two copies of the Ad itr. Thus, there are no homologous sequences between the vector construct and the helper plasmid. Vector is produced upon infection of the co-transfected cell with Ad. In this system the vector virions are free of contamination by wild-type AAV. However, it appears probable that Rep protein is required for site-specific integration and thus, these vectors do not integrate in any specific site. Under selection pressure in cell culture the vectors do integrate at a variety of sites, but whether such vectors used *in vivo* integrate or persist as episomes is uncertain. Nevertheless, use of such AAV vectors in animal models has led to long-term expression of the transferred gene. However, the possibility remains that more useful vectors might be developed that could have the property of site-specific integration. Although the questions such as whether such site specificity might have unanticipated pathology associated, or whether expression of the transferred gene might not be optimal at the site on chromosome 19, must be considered, site-specificity of integration remains the special attribute of AAV that first raised the possibility of its use as a gene vec-

Table 1. *Adeno-associated virus recombinants in application*

Gene	Promoter	Reference
α globin anti-sense	HSV tk	57
FACC	RSV-LTR	58
γ globin	γ globin/LCR site II	59
CFTR	AAV p5	60
HIV anti-sense	RSV-LTR	61

Abbreviations: CAT, cloramphenicol acetyl transferase; neo, neomycin phosphotransferase; FACC, Fanconi's anemia; CFTR, cystic fibrosis transmembrane conductance regulator; CMV, cytomegalo virus; HSV tk, herpes simplex virus thymidine kinase; SV 40, simian virus; RSV-LTR, Rous sarcoma virus long terminal repeat.

tor. Another question to be addressed is the possibility of increasing the genetic capacity of AAV vectors.

In spite of the limited size of the genome, a significant number of genes have been cloned into AAV vectors that have been used to infect cells *in vivo*. In addition to selectable markers such as neomycin phosphotransferase and chloramphenicol acetyl transferase, such genes as gamma globin (human), Fanconi's anemia, cystic fibrosis transmembrane conductance regulator and anti-sense RNA for HIV have been transferred into a variety of cells in culture. A number of such reports are listed in Table 1. Of particular interest are reports of transfer into cells that are not undergoing cell division. Such cells are transformed, and when examined are found to have the vector sequences integrated into the cell genome. However, to actually demonstrate integration it has been necessary to amplify the cells at some point and, thus, it is not completely clear that integration took place prior to amplification. The ability to transform stem cells would greatly heighten interest in AAV as a vector.

Prospective studies

Future areas of investigation of AAV will undoubtedly include continuing studies on the molecular biology of both replication and latency. The possibility of using AAV as a vector for gene therapy should increase interest in the normal interaction of the virus with the intact host and in the technical problems associated with application: packaging *in vivo* and *in vitro*, increasing the capacity of the vector, and assuring integration and, possibly, site specificity.

All nuclear DNA viruses cause persistent or latent infections. Of the known DNA viruses, AAV seems to have evolved a life cycle that emphasizes the latent component. Whether the site specificity of integration is an essential component, or merely the specific way that AAV has evolved, is not certain. If the proposed signal sequences (Rep-binding site, trs and/or M26 recombination enhancer) prove to be involved, the question of the function of these signals in the normal cell will arise. To date, the site of integration has been found to be transcribed at low levels in several tissues and to be present in cDNA libraries. However, the sequence does not correspond to any of the sequences contained in Genebank. Identification of the gene product might suggest whether or not there is a significant risk in having the virus integrate at that site.

In summary, AAV is an unusual virus whose ubiquity and potential utility make it an interesting area of study.

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Simian Virus 40 Assembly

INTRODUCTION

Despite the importance of SV40 in examining DNA folding and structure–function relationships of chromosomes in animal cells, the study of virion assembly has blossomed only recently. A number of major conceptual advances in understanding virus assembly have been developed over the past two decades from studies conducted on bacterial viruses. It appears that the assembly pathways of bacteriophages are in general quite strictly ordered and regulated (reviewed in Refs. 1 and 2). Some steps in assembly require protein processing, modification, and conformational changes; other steps in the process require specific recognition of assembly signals on the genome or intervention of proteins that are not themselves found in the completed virion but act as assembly devices and catalysts.[1,2] Among the "devices" that have been characterized are proteins that form transient scaffolds which direct the assembly of the precursor shell, or procapsid.[2] For example, during the assembly of the correctly dimensioned precursor shell of *Salmonella* bacteriophage P22, approximately 200 scaffolding protein subunits interact with about 420 coat protein subunits to form a double shelled particle with the scaffolding protein on the inside.[2] The actual DNA packaging event has also been a central issue and it has become clear that the bacteriophage DNA is condensed into

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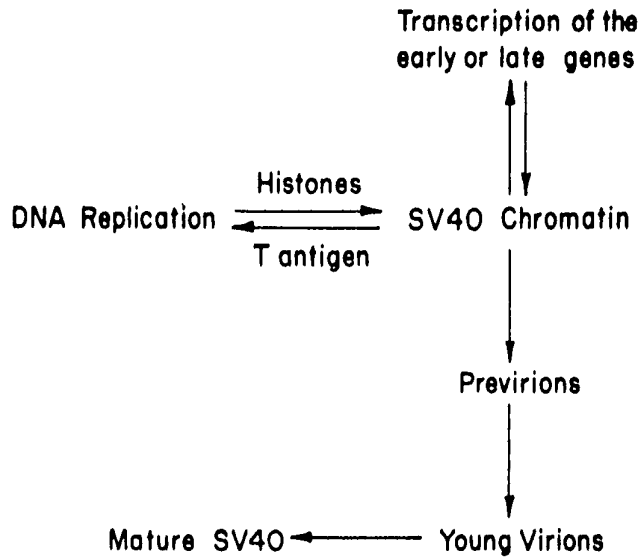

FIGURE 1 *In vivo*, SV40 chromatin is utilized for either DNA replication, gene expression, or virus assembly. The T antigen protein is required for the initiation of replication. During replication the cellular core histones fold the viral DNA into nucleosomes.

a preformed procapsid.[1,2] The scaffolding proteins exit through the coat protein lattice, presumably as the bacteriophage DNA enters the precursor shell, to take part in further rounds of the same process.[2]

In contrast, SV40 DNA is packaged by a stepwise process (Fig. 1). Like cellular DNA, the first step involves folding of the viral DNA by the most abundant chromosomal proteins, the histones, into nucleosomes formed by the wrapping of about 146–200 base pairs of DNA around octamers of core histones—two each of H2A, H2B, H3, and H4 (reviewed in Ref. 3). Because of its resemblance to cellular chromatin, the nucleoprotein complex formed between SV40 DNA and the histones is called a minichromosome, SV40 chromosome, or SV40 chromatin.[4]

The organization of DNA into nucleosomes is the first step in a sequential compaction process which eventually leads to the condensation of cellular DNA into metaphase chromosomes or SV40 DNA into virions. Paulson and Laemmli have identified several

nonhistone chromosomal proteins which form a scaffold from which chromosomal DNA emanates.[5] It is currently thought that the scaffolding proteins play a role in the higher order organization of cellular chromatin.[5] In the case of SV40, the viral proteins VP1, VP2, and VP3 are involved in packaging SV40 chromosomes in the course of virion maturation (reviewed in Ref. 6). Another SV40-coded protein called agnoprotein is not found in mature virions[7–9] but has been implicated in playing a role in virus assembly[8–11] or in controlling the expression of the viral late genes.[12,13]

THE SV40 ASSEMBLY PATHWAY

A central question raised by the researchers in the field has been the pathway whereby the minichromosomes are encapsidated in the course of virion maturation. Two general models have been proposed. In the first model, the viral chromatin is introduced into a preformed shell composed of VP1, VP2, and VP3 (reviewed in Ref. 6). In the second model, the shell assembly proceeds by the gradual addition of the capsid proteins to chromatin.[14–16]

The first model evolved from the finding that shells lacking DNA—empty shells—are isolated from lysates of infected cells banded to equilibrium in CsCl gradients.[6] Moreover, this model appears plausible because elegant studies have established that empty shells serve as precursors in the assembly of bacteriophages.[1,2]

Accumulating evidence indicates that the empty-shell model may not be true for SV40 assembly. Pulse-chase analysis has demonstrated that in the course of SV40 maturation, the 75 S chromatin is condensed by the capsid proteins forming 180 S previrions. These previrions are then converted to 220 S salt-labile young virions, which mature with time to yield salt-stable mature SV40[15–18] (Fig. 1). Moreover, two groups[17,18] have independently demonstrated that the empty shells isolated in CsCl[6] are the dissociation products of young and previrions in high salt. These results lead to the second model which postulates that the shell is built via the gradual addition of the capsid proteins to chromatin.[14,16] For the second model to be correct, it is necessary that the previrion as-

sembly be cooperative—that is, the propagation step of shell assembly must be much faster than the initiation event—because the putative intermediates which bridge the chromatin to previrions do not accumulate in cells infected with a wild-type SV40. From these cells one obtains primarily chromatin, at 75 S, and mature and young virions which cosediment at 220 S.[3] If the shell propagation step were rate limiting, nucleoprotein complexes sedimenting between 75 S and 220 S would have been detected in cells infected with a wild-type virus.

Our studies on SV40 temperature-sensitive assembly mutants support the second model. These mutants—classified as tsC, tsBC, and tsB[19,20]—synthesize altered forms of the major capsid protein VP1, which can function at the permissive temperature (33 °C) but not at the nonpermissive temperature (40 °C). The temperature-sensitive assembly mutants have the useful property that the assembly process can be examined both at the nonpermissive and permissive temperatures. From such experiments, one can, for example, identify the structural intermediates which accumulate in cells when the initiation or propagation steps of assembly are impaired at 40 °C. In addition, by shifting the temperature from nonpermissive to permissive, one may be able to determine whether these structural intermediates can be used in virion assembly *in vivo*.

We have found that at 40 °C, the 75 S chromatin accumulates in cells infected with the tsC mutants because virion assembly does not initiate.[21] In contrast, in tsBC-infected cells, the capsid proteins associate with the minichromosomes to form capsid–chromatin complexes which sediment from 100–140 S.[23] These results indicate that the capsid proteins may interact directly with the minichromosomes during virion assembly. In cells infected with the tsB group of mutants, the block in virion assembly results in the accumulation of the 75 S chromatin and semiassembled virions which sediment as a broad peak from 100–160 S.[24] Under the electron microscope, the semiassembled virion appears as a shell-like structure attached to the SV40 chromosome.[21] Biochemical analysis has revealed that the shell consists of partially polymerized capsid proteins held to the chromatin by electrostatic interactions.[24] Assembly in tsB-infected cells appears to be blocked at various stages of the shell propagation step after virus formation has been initiated. This finding provides strong support for the hypothesis that the shell is formed by the gradual addition and organization of the capsid proteins around SV40 chromatin.[22,24]

Granted that the SV40 assembly proceeds via the addition of the capsid proteins to chromatin, several questions come immediately to mind. For example: How is the assembly initiated? Are the capsid proteins added randomly to the minichromosomes, or do they bind initially at preferred sites on SV40 DNA from which shell polymerization propagates? Does the structure of the viral chromatin change prior to or in the course of virion assembly? And, what factors are involved in forming shells of proper size and dimensions around the minichromosomes?

INITIATION AND PROPAGATION OF SHELL ASSEMBLY

Hsu and co-workers used micrococcal nuclease digestion analysis to compare the structure of the 75 S chromatin with that assembled into the 180 S previrion.[25] Micrococcal nuclease cleaves chromatin in the regions not protected by the core histones.[26] When DNA is isolated from partially digested chromatin and analyzed by gel electrophoresis, a series of bands is obtained.[26] The sizes of these DNA fragments reflect the nucleosome spacing periodicity[26] or repeat length (reviewed in Ref. 27). Coca-Prados and Hsu[25] showed that the nucleosome repeat length for the previrion chromatin (211 ± 14 base pairs) was larger than that observed for the 75 S chromatin (194 ± 8 base pairs). This difference might arise from binding of VP3 to DNA regions which link the nucleosomes to yield assembly-committed minichromosomes. These chromosomes may then be coiled by VP1 to form a toroidal superhelix as proposed, for example, by Keller and co-workers.[28] Further compaction of the superhelix would result in virion formation.[28]

Another model, which I favor, involves the binding of a cluster of the major capsid protein VP1 to the VP3-minichromosome complex, possibly at a preferred or selected site, to form an assembly-initiation complex. In the propagation phase of the assembly process, the incoming capsomers would be preferentially incorporated relative to this nucleated complex, resulting in radial spreading of the shell around the viral chromatin (Fig. 2). This type of shell-assembly propagation may be regulated by a conformational

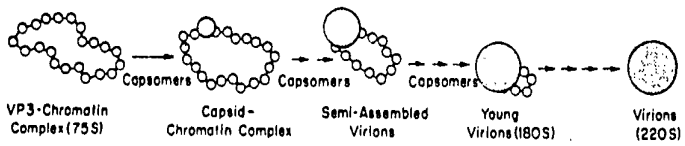

FIGURE 2 SV40 assembly may initiate by binding a cluster of the major capsid protein to selected sites on the VP3-minichromosome complex. In the propagation phase, the capsomers would be incorporated onto the initiation complex, resulting in the formation of the capsid around the chromatin.

switching mechanism described, for example, in the assembly of flagella, microtubles, and bacteriophage precursor shells (reviewed in Ref. 29). In such a mechanism, the polymerization is controlled by the existence of two conformational states of the protein subunits. When free in solution, the subunits are inactive and do not spontaneously polymerize. However, when bound to a growing structure, or to an initiation complex, the subunits are activated. This generates the growing complex for the binding of the next subunit. Thus, the rate of growth is limited only by the time it takes for the subunits to switch their conformation to the polymer form at the site of attachment.[29-32]

A conformational switching model, if applied to SV40, predicts that the virion assembly process must be highly regulated. The term regulation may include switching the capsomer structure from "inactive" to "active" forms in the course of shell polymerization, the location of initiation and termination signals on the viral chromatin, protein modification such as phosphorylation and acetylation, participation of auxiliary protein factors—to direct the formation of a shell with proper dimensions around the chromatin—and feedback loops linking virion assembly to processes which control DNA replication and transcription.

ASSEMBLY OF SHELL WITH PROPER SHAPE AND DIMENSIONS

If SV40 shell assembly proceeds by the direct addition of VP1, VP2, and VP3 to the minichromosome, it is reasonable to ask whether the information for constructing a shell with proper dimensions is specified by the contour length of the viral chromatin or mediated by an auxiliary protein such as the SV40 agnogene product.

The agnoprotein is encoded within the leader region of some of the mRNAs used in the synthesis of virion proteins.[7,9,33] It has, therefore, also been named leader protein 1 (LP1).[34] The leader becomes attached to the coding regions of VP2 and VP1 by alternative processing pathways of primary mRNA transcripts (reviewed in Ref. 6). In addition to being coexpressed with the viral proteins, the agnoprotein shares another common property with the scaffolding protein of bacteriophages in that it is not found in mature virions.[7-9]

We have found that in contrast to mutants of bacteriophage scaffolding proteins,[2] SV40 virions with proper shape and dimensions are produced in cells infected with LP1-deletion mutants.[11] However, our data obtained from both steady-state and pulse-chase radiolabeling experiments have demonstrated that in cells infected with the agnogene deletion mutants, virions are produced more slowly than in cells infected with the wild-type parental strain, wt800.[11] These findings, together with results showing that comparable levels of VP1 are synthesized both in wild-type and in deletion-mutant-infected cells,[11] strongly suggest that LP1 may play a role in expediting virion assembly rather than being involved in shaping the shell structure around the minichromosomes.

Since a protein comparable to bacteriophage scaffolding proteins has not been found for SV40, I propose that the size and structure of SV40 chromatin are involved in directing the assembly of shells with proper dimensions. That is, I can envisage that an SV40 chromatin, with a correct higher order structure, could provide the scaffold needed for assembly of an icosahedron, 420 Å in diameter. My hypothesis is compatible with the finding that DNA molecules which are either 2% longer or 5% shorter than the SV40 genome will not produce viable virus.[35] We have several experiments in progress to test this hypothesis more directly.

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Through Proper Channels: A View of General Anesthesia

A SEDUCTIVE TOPIC

The study of general anesthesia is alternately exhilirating and sobering. An understanding of anesthesia promises insights into that most difficult of territories, consciousness. Musings appropriate to this research area lead naturally into manic flights of fancy. However, one is rudely jolted back to reality by the ever-present difficulties of trying to define the neurobiological underpinnings of a behavioral phenomenon, when the behavior itself is poorly categorized. It is, therefore, a difficult area of research. However, the appeal of this research area is multi-faceted. On a visceral level, the phenomenon of general anesthesia evokes what may be primal concerns regarding "self," and loss of "self." Certainly, it is a remarkable phenomenon to willingly allow oneself to be cut open by a surgeon's knife; yet the introduction of structurally simple molecules of a general anesthetic results in just this outcome. The fascination with the problem parallels similar fascinations with hypnosis, hallucinogenic drugs, and the related areas of sleep and dreaming. All offer the seductive promise of a much better understanding of the nature of the human will, by the knowledge of how the normal reins of the mind are altered, in the case of anesthesia, by a large array of superficially unrelated (at least structurally) pharmacological agents. It is not surprising that the initial introduction of general anesthetics, only a little more than one hundred years ago, evoked such an animated response from those concerned with our spiritual well-being, such as the clergy.[1] The study

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